THE BRITISH JOURNAL OF
EXPERIMENTAL PATHOLOGY

VOL. XIV DECEMBER, 1933 No. 6

OBSERVATIONS ON ANTI-PHAGE SERA. I: "THE PERCENTAGE LAW".

C. H. ANDREWES AND W. J. ELFORD.

From the National Institute for Medical Research, Hampstead, London.

Received for publication August 16th, 1933.

THE literature which deals with the interaction between bacteriophages and their neutralizing antibodies is both extensive and contradictory. We shall not attempt to review it, as this has recently been done by Schultz, Quigley and Bullock (1929). Many of the contradictions we feel are only apparent; workers have not sufficiently realized that facts which are true about one phage do not necessarily apply to all phages.

There is general agreement upon certain facts. All workers find that phage can act as an antigenic entity, and that the neutralizing antibodies obtained by immunizing animals are quite distinct from antibacterial antibodies in the serum. Further, the antibodies are thermo-stable, and their neutralizing action is effective in the absence of complement. Neutralization takes place slowly, but is more rapid at higher temperatures.

Concerning other points there is still dispute. Amongst the workers quoted in the review already mentioned, support may be found for the view that the reaction does—or does not—obey the laws of multiple proportions; that it is—or is not—a reversible one; that the Danysz effect is—or is not—demonstrable. Of particular interest in relation to the facts we propose to describe are the statements (Wagemans, 1923; Arnold and Weiss, 1924; Schultz, Quigley and Bullock, 1929) that the phage and antiphage interact in constant proportions. None of the authors making this statement have varied the proportions of serum and phage over other than a very narrow range, nor do they appear to have studied the course of neutralization curves with various phage-antiphage mixtures. In fact, though their conclusions are widely quoted, we cannot find records of any protocols which would justify the drawing of any such conclusions as theirs.

SPECIFICITY OF ANTIPHAGE SERA.

We have found, as Burnet (1932) and others have done, that many anti-phage sera are strictly specific. On the other hand, some cross-reactions occur, and by the study of these it is possible to arrange many phages in

serological groups (*cf.* Burnet, 1932). For instance, C36, the coli-phage most studied by us in this and earlier papers (Andrewes and Elford, 1932 ; Andrewes, 1932) falls into one group with three others attacking intestinal bacteria (S18, C38, M) ; antisera prepared against C36 and S18 will neutralize all the phages in this group. We have found, however, that both the anti-C36 and anti-S18 sera are active against their homologous phages in about ten times as high a dilution as against the other members of their group.

EXPERIMENTAL METHODS.

The phages we have particularly studied have all been described in previous papers by ourselves or Burnet (Elford and Andrewes, 1932 ; Burnet, 1932). All except *Staph. K* attack various strains of *B. coli* and *B. dysenteriæ*. Three have been particularly chosen as representative phages of very different size groups ; C13 is a small one, C36 a moderate-sized one and C16 a large one (*cf.* Elford and Andrewes, 1932). C16, whose size has been estimated since the paper referred to, has a diameter estimated at 50–75 mμ., approximately the same as that of *Staph. K*. Phage titres have been determined by plating dilutions on 2 per cent. agar and counting plaques. It is particularly important in quantitative work with antiphage sera to use standard plates ; accordingly, in most experiments we poured 20 c.c. of agar into 8·5 cm. plates. Plates were well dried in the incubator, both before and after spreading.

Antisera to C36, S18 and *Staph. K* were prepared as described earlier (Andrewes, 1932). For antisera against C16 and C13 we are greatly indebted to Dr. F. M. Burnet ; he has described the method of preparation of those (Burnet, 1932). Certain phages seem to give rise regularly to high-titre sera ; others are less satisfactory. Anti-C36 serum, that most studied by us, was only moderately active ; neutralizing power could, in most batches, be detected in a dilution of 1 : 1000. Some lots of anti-C16 serum, on the other hand, showed demonstrable activity when diluted 1 : 100,000.

FORM OF THE NEUTRALIZATION CURVE.

Light may be thrown on the phage-antiphage reaction by studying the form of the neutralization curve obtained when the amount of phage surviving in a phage-serum mixture, incubated at 37° C., is plotted against time. Such curves are seen in Figs. 1 and 2, the phage being plotted on a logarithmic scale. It will be seen that, particularly with stronger sera, the curve is very steep at first, but soon flattens out. With more dilute sera the slope is gentler and more uniform. When the curves have begun to flatten out, neutralization continues more and more slowly during about 48 or 72 hours, after which no further action is demonstrable. With more dilute systems it may go on even longer, but this is hard to be sure of ; for after some days at 37°, the titre of phage may fall off even in the absence of antiserum. Results similar to the above have been recorded by Wagemans (1923) and by Schultz, Quigley and Woolsey (1931).

The last-named authors have maintained that the curves they obtained

fit very well with those describing the velocity of an adsorption reaction. They suggest that in the later stages the speed of the reaction is progressively slowed up because particles capable of interacting are becoming so few that the chances of a collision are increasingly remote; hence the flattening out

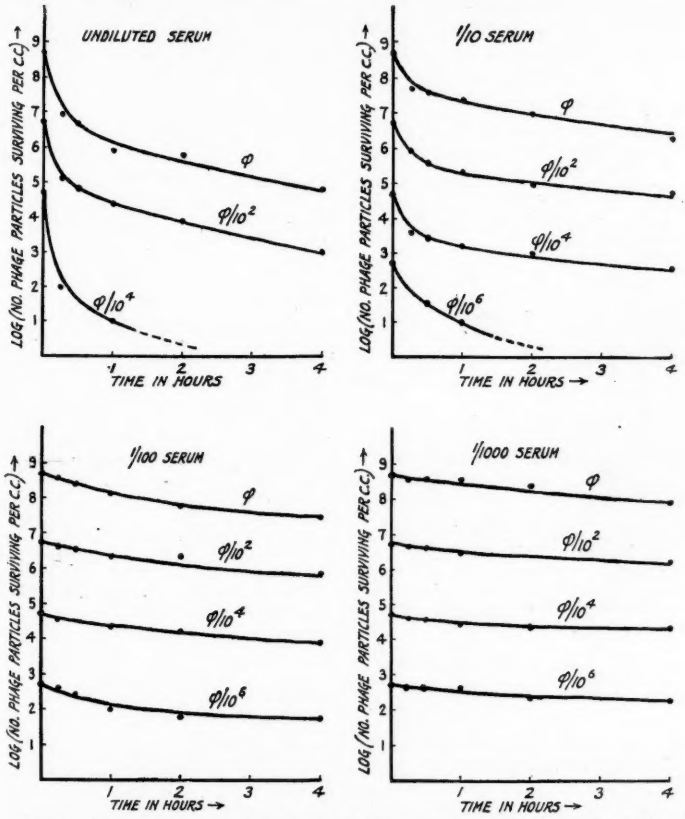


FIG. 1.—Graphs showing the neutralization of various concentrations of phage C36 by given dilutions of its antiserum. ϕ = undiluted phage = 5×10^8/c.c.; $\phi 10^2$ = phage diluted 1 : 100; $\phi 10^4$ = phage diluted 1 : 10,000; $\phi 10^6$ = phage diluted 1 : 1,000,000.

of the curve. We do not wish to dispute that adsorption may play a part in the phage-antiphage reaction, but the facts we shall record later show that Schultz's explanation of the flattening out of the curve is inadequate. An account of one experiment will suffice for the moment to show this.

A phage-serum mixture was incubated at 20° C. for 20 hours; by this time the neutralization curve would be almost flat. A test showed that 1250 phage particles per c.c. remained unneutralized. The mixture was filtered

through a membrane which could hold back phage but not antibody. The filtrate was mixed with an equal volume of phage containing about 2500 particles per c.c.; this mixture should thus contain in each c.c. as much phage and half as much antiserum as was present in the original mixture before filtration, and if Schultz's explanation was correct, the neutralization curve should be nearly flat. On the contrary, it was found to be precipitously steep, all the phage being neutralized within 5 minutes. The slowing-up of the reaction has, therefore, to be accounted for in some other way.

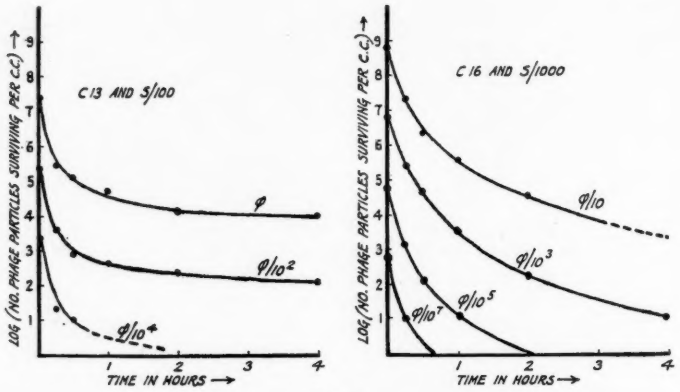


FIG. 2.

Curves showing neutralization of various concentrations of C13 phage by a 1 : 100 dilution of its antiserum at 37·5° C. ϕ = undiluted phage = $2{\cdot}5 \times 10^7$/c.c.; $\phi 10^2$ = phage diluted 1 : 100; $\phi 10^4$ = phage diluted 1 : 10,000.

Curves showing neutralization of various concentrations of C16 phage by a 1 : 1000 dilution of its antiserum at 37·5° C. ϕ 10 = phage diluted 1 : 10 = 6×10^8/c.c.; $\phi 10^3$ = phage diluted 1 : 1000; and so on.

NEED FOR EXCESS ANTIBODY.

Early in our investigations it became clear that complete neutralization of phage only occurred in the presence of a large excess of antibody. In one experiment undiluted phage C36 was mixed with the smallest amount of antiserum which would completely neutralize it in 72 hours at 37° C.; this was an equal volume of antiserum diluted 1 in 3. If phage and antibody had combined according to the multiple proportion law, this should have proved an exactly balanced mixture. When, however, the system was examined at the end of 72 hours, it proved to be almost or quite as potent in its neutralizing properties as it was originally. Thus, the apparently "just neutral" mixture must actually have contained a large excess of antibody. This finding was not in itself very astonishing, for it must be remembered in comparing the reaction with a toxin-antitoxin union that one can readily detect unneutralized phage when this represents only a millionth of that originally present; residual toxin cannot, of course, be recognized

with anything like such delicacy, and it might well be that a large excess of antitoxin would be needed to neutralize 99·9999 per cent. of toxin.

But our later studies led us to believe that a large excess of antiphage antibody was needful, not only to produce complete inactivation of phage, but to produce any demonstrable neutralization at all. Our difficulties in absorbing out neutralizing antibodies with a large excess of phage pointed in this direction. One of our poorer samples of anti-C36 serum was not demonstrably active in more than a 1 : 100 dilution even against phage diluted $1 : 10^6$; 1 : 100 serum could only incompletely neutralize this dilution of phage. We therefore argued that if this dilution of serum were mixed with *undiluted* phage there should be a colossal excess of phage present. This mixture was accordingly made, incubated 30 minutes at 40° C. and then left overnight on ice. It was then filtered through a 55 mμ. membrane to remove the neutralized and the still plentiful unneutralized phage, and its activity was compared with a control lot of unabsorbed serum; its neutralizing power was found to be very little diminished.

Very many other similar absorption experiments were carried out with antisera to C36, S18 and C16. Both homologous and heterologous phages were used for the absorptions. Frequently we failed to effect any absorption at all; when we used the strongest available phage and the weakest recognizable dilution of serum we could at best remove a little antibody; only with C16 and its antiserum was the absorption at all near complete. Burnet (1933, *a*) has since devised a technique which enables him to absorb with much heavier doses of phage, and with this method antibody absorption has been successful. Our point at the moment is that our methods, which on paper looked so certain to succeed, proved so disappointing.

THE " PERCENTAGE LAW ".

We were, therefore, led to inquire whether, in our attempted absorptions, we were really employing such vast excess of phage as we supposed. When we wished to detect the presence of dilute antibody, we had naturally tested it against very dilute phage; perceptible neutralization by dilute antiserum of phage a million times as strong was hardly to be expected. The actual test had, however, to be made. A 1 : 100 dilution of an anti-C36 serum was taken; in 4 hours this neutralized 95 per cent. of phage, diluted 1 : 1,000,000. To our intense astonishment it also neutralized 95 per cent. of the undiluted phage. We studied the matter fully and found, in brief, that over a very wide range a given dilution of serum neutralized in a given time an approximately constant percentage of phage, *however much phage there was present.* This phenomenon we refer to for convenience as the " percentage law ".

The rate of neutralization of phage by antiserum was also found to be practically independent of the amount of phage present. In Figs. 1 and 2 are shown a series of curves depicting the neutralization of the phages C36, C16 and C13 by their respective antisera. The phage-serum mixtures under study were shaken in a mechanical shaker in the hot-room (37° C.); samples were removed at intervals, chilled to 0° C., and suitable dilutions in broth plated on agar. The antiserum-dilutions were all made in normal rabbit

serum. Each group of curves in Fig. 1 shows the rate of neutralization of different dilutions of phage by one serum dilution; the titre of surviving phage is plotted on a logarithmic scale. It will be evident that each antiserum-dilution inactivates a roughly constant percentage of phage in a given time, for the various curves in each group are approximately parallel. The somewhat anomalous curves obtained when neutralizing dilute phage by strong (undiluted and 1 : 10) antiserum are probably explained by the fact that these systems, when plated on agar, could not be diluted, so that the reaction was not immediately arrested, and could proceed for a while on the agar surface before the phage became adsorbed by susceptible bacteria.

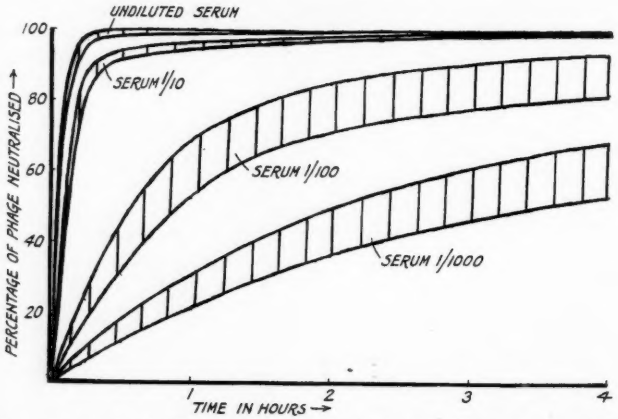


FIG. 3.—The percentage neutralization of C36 phage by various dilutions of its antiserum at 37·5° C. The zones crossed by the tie bands indicate the limits within which the experimental points for different phage concentrations were found to lie.

The "percentage law" was also found to hold when C36 was incubated with anti-S18 serum, a serum prepared against a member of the same serological group; and in Fig. 2 are shown similar curves for the inactivation of phages C13 and C16 by homologous antisera. Curves for only one serum dilution are shown in each instance, but the data for other dilutions conformed to the law as well as they did in the case of C36. The forms of the curves for C16 will be noted to be rather different from the others, the slowing up of the reaction being more gradual.

The "percentage law" is illustrated in another way in Fig. 3. The percentage neutralization curves of various phage dilutions by any one strength of serum are there shown all to lie within comparatively narrow zones.

Dilution of an antiserum beyond a certain point seemed to deprive it of all activity against even dilute phage. Thus, anti-C36 serum 1 : 100 had considerable neutralizing power against the strongest phage; yet 1 : 1000 serum had scarcely any activity however long it was incubated with phage a million times as weak. Similarly, 1 : 10,000 anti-C16 serum could inactivate

the greater part of undiluted phage ; but 1 : 100,000 serum was practically ineffective against the weakest dilution of C16. It appears, then, that a serum in its weakest effective concentration can neutralize strong phage as readily as it can dilute phage ; a large excess of antiserum must, therefore, be available in the mixture containing dilute phage. But the similarity of the neutralization curves for all the phage-dilutions suggests that the reaction is, in each case, governed by similar quantitative relations. This is easy to understand only if excess antibody is present in *all* the mixtures, including those containing undiluted phage. The tentative conclusions reached as a result of the abortive attempts at antibody absorption are thus confirmed. We shall discuss later

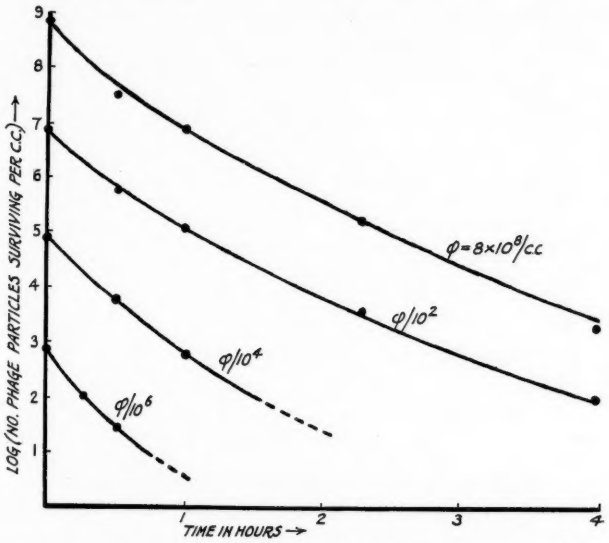


FIG. 4.—Curves showing the course of inactivation of various concentrations of C36 phage by 0·05 per cent. formaldehyde.

possible reasons for this dependence of neutralization on the presence of excess of antibody in the system. It is of interest that the inactivation of C36 by dilute formaldehyde was also found to obey the " percentage law " (see Fig. 4).

WHY DOES SOME PHAGE ESCAPE NEUTRALIZATION ?

If all the systems studied contain excess antibody, it is hard to understand why there always remains a percentage, constant for each serum-dilution, of unneutralized phage. Several possible explanations have to be considered.

I. Is there a Mass Law Equilibrium ?

We may first inquire whether the systems reach an equilibrium according to the mass law of the type : Phage + antibody $\rightleftarrows$ phage-antibody complex.

The presence of complex would then retard the completing of the reaction. We devised experiments of several different types with the object of demonstrating an effect of the postulated complex on the speed of the reaction. No such effect could be shown. On one occasion we mixed C36 (diluted $1 : 10^4$) with 1 : 100 antiserum. The neutralization curve was plotted. After an hour a second equal dose of C36 was added ; the curve of its neutralization was the same as after the first dose. At the time it was added the curve of neutralization of the first sample was flattening out ; if this flattening out were due to the retarding influence of a complex, the second instalment of phage should not have been inactivated as rapidly as we found. Incidentally, we may note that the experiment done in this way gives no evidence of a Danysz effect. Further evidence against the idea that a mass law type of equilibrium was involved was obtained as follows : a phage-serum mixture was filtered through a membrane which would retain phage and phage-antibody complex, but let through antiserum. When nine-tenths had passed through the remaining tenth was removed and made up to its original volume. If the phage serum reaction were governed by an equilibrium of the type $\frac{\text{(phage)} \times \text{(antiserum)}}{\text{(complex)}} = \text{constant}$, the removal by filtration of the free antiserum (of which there was plenty) should have permitted some dissociation of the complex ; we could find no evidence of such dissociation in several attempts—the phage titre was, if anything, lower than that of a control mixture.

II. Does the Method of Making the Mixtures Affect the Outcome.

It seemed possible that phage, which, in the early stages of the reaction had received a partial coating with antibody, might, under certain conditions, reach a state—perhaps through change in its surface properties or as a result of aggregation—in which the more thorough coating necessary for complete neutralization was hard to achieve. Thus, the phage particles which escaped inactivation, by chance, in the early stages of the reaction might fail to be neutralized at all. To test this possibility, we shook phage C36 with 1 : 100 antiserum for an hour at 37°, and then added an equal volume of 1 : 10 serum to the mixture. The neutralization curve was exactly the same as that of a control lot of untreated phage mixed with 1 : 10 serum. Other experiments, on similar lines, confirmed this result. There was thus no evidence in support of this second hypothesis ; phage which survived the early stages of contact with dilute antiserum was normally susceptible when rather stronger antibody was added later.

III. Do Phage Particles Vary in their Resistance to Antibody ?

A natural variation in the resistance of phage particles to antibody would seem an obvious explanation of the "percentage law" ; a definite percentage of phage would thus be resistant to each antibody dilution. This explanation of the form of the neutralization curve has been put forward by d'Hérelle (1926). A similar problem, that of natural variation in resistance of individual bacteria, is familiar also to students of disinfection. In seeking to test this

third hypothesis, we selected plaques of C36 which had survived treatment with antiserum and tested their descendants. These were normally susceptible, and there was thus no evidence of an *inheritable* serum-resistance.

We next tried to correlate resistance to antiserum with other possibly varying properties of phage particles :

(*a*) Undiluted phage was heated for an hour at 65° C. ; 99·8 per cent. was inactivated. The surviving 0·2 per cent. showed normal resistance to antibody.

(*b*) Phage diluted 1 : 100 was absorbed three times with heat-killed rough dysentery bacilli (Flexner, W.R.). Each 1 c.c. quantity of phage was absorbed with 10^{10} organisms for 20 minutes at 48° C. The 4 per cent. of phage which remained unabsorbed showed normal resistance to antibody. Schlesinger has produced evidence (1932) that a certain percentage of some strains of phage is unabsorbable by bacteria. We found no evidence that absorption-resisting phage was also serum-resistant.

(*c*) No difference could be detected between the neutralization curves of freshly-prepared phage and of a batch which had been kept in the cold for 24 days. We tested this point, because Capt. S. R. Douglas had told us of some unpublished work of his with bactericidal serum, active against *B. typhosus*. He had found, as we had with phage, that the percentage of organisms killed by a given concentration of serum was, over a wide range, relatively independent of the numbers of organisms present. He had found, however, that the percentage of bacilli killed differed greatly according to whether a freshly isolated or an old laboratory culture was used.

(*d*) C36, grown on *B. coli*, is active against rough dysentery bacilli (Flexner, W.R.), but only to about one four-hundredth of the titre at which it will attack *B. coli*. The explanation of such a finding, which is very familiar to workers with phage, is still obscure. It is conceivable that the phage particles are not uniform, and that only 1 in 400 can attack the rough W.R. dysentery. With this possibility in mind, we incubated phage with antiserum under various conditions, and tested the mixture ; the ratio titre against *B. coli* : titre against W.R. remained unchanged. C36 grown on W.R. dysentery was similarly tested. With such phage the ratio in question, instead of being 400 : 1, is of the order of 1 : 5, but here again the supposed serum-resistant units gave the same ratio as control phage. Tests were made both with sera prepared against coli-grown phage and W.R.-grown phage.

Our failure to correlate resistance to antiserum with other supposedly varying properties of phage particles does not, we feel, exclude the possibility that variation in serum-resistance may explain the form of the neutralization curve and the percentage law. It is, however, an explanation of which direct proof is very hard to obtain.

EFFECT OF PHAGE ULTRA-FILTRATE.

Burnet (1933, *b*) has shown that an ultra-filtrate of phage—a filtrate through a membrane which retains the phage itself—has some power of binding anti-phage antibody. He believes that this ultra-filtrate contains an antigen or haptene separable from the phage itself. We can confirm this result as regards

C16, but the binding effect is small compared with that of phage itself, and we do not, therefore, think it likely that this agent in a free state can play any large part in determining the quantitative relations between phage and antibody.

DISCUSSION.

We are inclined to believe that the " percentage law " finds its explanation in a lack of uniformity of phage particles. These, as d'Hérelle (1926) supposed, seem to have varying resistance to neutralization by antiserum.

Further discussion of the mechanism of the interaction of phage and anti-phage is reserved to the end of the following paper on " Properties of Incompletely Neutralized Phage ".

SUMMARY.

1. The neutralization of three phages (C13, C16, C36) does not follow the multiple proportions law.

2. On the contrary, neutralization is only demonstrable in the presence of a large excess of antiserum.

3. A given strength of antiserum neutralizes, over a wide range, a definite percentage of phage, irrespective of the quantity of phage present.

REFERENCES.

ANDREWES, C. H.—(1932) *Brit. J. Exp. Path.*, **13**, 85.
Idem AND ELFORD, W. J.—(1932) *Ibid.*, **13**, 13.
ARNOLD, L., AND WEISS, E.—(1924) *J. Infect. Dis.*, **35**, 505.
BURNET, F. M.—(1932) *J. Path. Bact.*, **36**, 307.—(1933, *a*) *Brit. J. Exp. Path.*, **14**, 93.—(1933, *b*) *Ibid.*, **14**, 100.
D'HÉRELLE, F.—(1926) ' The Bacteriophage and its Behaviour ', translation by G. H. Smith. Baltimore (Williams & Wilkins).
ELFORD, W. J., AND ANDREWES, C. H.—(1932) *Brit. J. Exp. Path.*, **13**, 446.
SCHLESINGER, M.—(1932) *Z. Hyg. InfectKr.*, **114**, 136.
SCHULTZ, E. W., QUIGLEY, J. S., AND BULLOCK, L. T.—(1929) *J. Immunol.*, **17**, 245.
SCHULTZ, E. W., QUIGLEY, J. S., AND WOOLSEY, E. L.—(1931) *Ibid.*, **20**, 149.
WAGEMANS, J.—(1923) *Arch. Int. Pharmacodyn. Ther.*, **28**, 181.

OBSERVATIONS ON ANTI-PHAGE SERA. II: PROPERTIES OF INCOMPLETELY NEUTRALIZED PHAGE.

C. H. ANDREWES AND W. J. ELFORD.

From the National Institute for Medical Research, Hampstead, London.

Received for publication August 16th, 1933.

THE following paper describes experimental findings which suggest that a bacteriophage treated with antiserum under certain conditions may have its properties altered in very definite ways even though its ability to attack susceptible bacteria has not been removed. Such phage is referred to as

"incompletely neutralized". The findings will then be discussed in relation to those described in the preceding paper. The phages studied were, in the main, the same as those in that paper.

EFFECT OF ANTIBODY ON PLAQUE SIZE.

D'Hérelle, and also Seiffert (1923–24), have claimed that antiserum reduces the size of the plaques which phage produces on agar, but other workers have denied this. Both statements are correct, for homologous immune serum greatly reduces the plaque-size with some phages (*e. g.* C36), but hardly does so at all in other instances (C13, C16). Phage C36 was mixed with various dilutions of antiserum, and after an hour's incubation at 37° C. the mixtures were plated out on 2% agar. After overnight incubation, 20 plaques on each plate were measured and their average diameter estimated. The results shown in Table I were obtained.

TABLE I.—*Effect of Antiserum on Plaque-size.*

	Control (no serum).	With serum.				
		Undiluted.	1:10.	1:100.	1:1000.	1:10,000 or over.
Average diameter of plaque in mm.	0·75	0·25	0·38	0·54	0·68	0·77

The final dilution of serum spread on the plate did not affect the result. A mixture with strong serum still produced very small plaques when it had been diluted 10^4 times before being spread. Phage obtained from a small plaque gave rise on subsequent test to plaques of normal size; no permanent change in the character of the phage had been produced.

The size of plaque produced by any one phage depends largely upon the time during which it has been able to spread outwards before the bacteria on the plate have ceased to multiply, thus becoming insusceptible to lysis. If a plate is sown with organisms and incubated for some hours before being sown with phage, the available period during which phage can spread and plaques can grow is reduced. Thus, C36 cannot produce visible plaques on agar which has been sown with *B. coli* more than 7 hours beforehand; if the interval between the sowing of bacilli and of phage is only 2 hours, the plaques are all very small.

The available data combined to suggest that serum-phage mixtures produced small plaques because the phage in such mixtures only began to multiply after a considerable delay. Experiment showed that this was indeed the case. We plotted the rate of increase of phage in phage-serum mixtures incubated in test-tubes along with *B. coli*; the delay was very slight as compared with control tubes. But it occurred to us that if only one phage particle out of fifty escaped neutralization, it would be able to multiply so rapidly that in an hour its progeny would be sufficiently numerous to obscure completely the fate of the other 49. This type of experiment was, therefore, deemed unsatisfactory. We accordingly used a modification of a technique described by Burnet (1929). The essential feature of this is that the system

under study is divided into a large number of small portions, the *whole* of each of these portions being tested at the desired time-intervals.

We incubated together equal volumes of undiluted C36 and antiserum for 50 minutes at 37°; the mixture was then diluted 10^4 times and seeded with *B. coli* in the logarithmic stage of growth. We then placed on the bottom of each of 5 empty Petri dishes eight 0·02 c.c drops of the mixture. We had so arranged the quantities of phage and serum that each drop, when pipetted off as completely as possible and spread on agar, produced approximately 7 plaques. The Petri dishes were covered up, a sheet of sterile dental rubber being placed between the two halves of the dish to minimize evaporation. The drops in one dish were tested at once, and the other plates were incubated and removed for testing at appropriate intervals. The results are shown in Table II. Clearly, no multiplication has occurred in the first $1\frac{1}{2}$ hours.

TABLE II.—*Delay in Multiplication of Phage in a Phage-serum Mixture.*

Time of test.	Drops. 1.	2.	3.	4.	5.	6.	7.	8.	Average.
Immediate	10	8	3	13	6	3	11	4	7
$1\frac{1}{2}$ hours	3	5	0	7	13	3	16	10	7
$2\frac{1}{4}$,,	16	Almost C	17	12	5	140	13	19	..
3 ,,	C	C	C	30	14	28	8*		..
$3\frac{3}{4}$,,	C	C	C	C	C	C	C	C	..

Figures refer to numbers of plaques on plates spread with drops of mixture.
C = Complete lysis: Innumerable plaques. * Run together.

After $2\frac{1}{4}$ hours there is a significant increase in only a quarter of the drops, and since each of those doubtless contained a number of particles to start with, the percentage of particles which have started to multiply in this time must be very small. In a control experiment, drops of dilute phage without serum were similarly studied; in this instance there was only an average of one phage particle in each drop. Yet enormous multiplication had occurred within an hour in every drop in which phage was present at all. The evidence that phage in a mixture with antiserum only begins to multiply after a delay seems thus to be incontestable.

ADSORPTION OF INCOMPLETELY-NEUTRALIZED PHAGE.

Phage (C36) producing small plaques after treatment with antiserum presumably consists of phage particles which have their properties changed but which are not completely neutralized. We found that such phage was adsorbed in a normal manner by susceptible bacteria.

FILTRATION OF INCOMPLETELY-NEUTRALIZED PHAGE.

Freshly made mixtures of phage C36 with fairly concentrated antiserum were found to give inactive filtrates when 5 c.c. were passed through an L. 2

candle. Undiluted phage was rendered non-filterable by one batch of antiserum diluted 1 : 100, but most lots of serum were only effective up to 1 : 10 dilution. These dilutions of serum were incapable of completely neutralizing the phage even in 72 hours ; there was thus a contrast between the amount of phage which could be neutralized and the amount which could be rendered non-filterable. In order to show the effect of 1 : 10 or undiluted antiserum on the filterability of phage, no incubation of the phage-antibody mixture was necessary ; phage filtered immediately after mixing with serum was completely retained by the candle. Phage unmixed with antiserum, although producing many fewer plaques on agar than the unfiltered phage-serum mixtures under study, was found to be scarcely diminished in titre when 5 c.c. quantities were filtered. Its filterability was also unaffected by agglutinating antisera against *B. coli* and by antisera prepared against heterologous phages.

The filtration of S18 was similarly stopped by a dilution of homologous antiserum which could not completely neutralize it ; but with *Staph. K* phage and its antiserum, this differential action of certain strengths of serum was not demonstrable.

Similar results were obtained when we used Gradocol membranes (Elford, 1931) instead of candles. C36 is normally held back by a membrane having an average pore size of 60 mμ. In a mixture with undiluted or 1 : 10 antiserum, however, filtrates through 220 mμ. membranes were usually sterile. It was difficult to filter mixtures through these membranes, for although phage and antibody passed through readily when separate, yet they quickly blocked the pores of the membranes when combined in mixtures.

d'Hérelle (1921) obtained evidence of agglutination of phage by antiserum by comparing titres in the upper and lower layers of a phage-serum mixture after centrifuging. Recently Burnet (1933, *b*) and Schlesinger (1933) have obtained more direct evidence of agglutination of phages by homologous antisera. There seems no reason, therefore, for refusing to draw the obvious conclusion that the phage particles in our experiments failed to filter because they had been agglutinated by the antisera. Clearly, however, the process of agglutination does not necessarily involve the inactivation of the phage. We should not wish to deny that some part might be played also by increased " stickiness " of the phage-antibody complexes resulting in greater adsorption on to the walls of the filter.

APPARENT RE-ACTIVATION OF NEUTRAL MIXTURES TESTED IN BROTH.

Otto and Munter (1922) first recorded reactivation of neutral phage-antiphage mixtures by dilution. Most other workers (Muckenfuss, 1928 ; Schultz, Quigley and Woolsey, 1931 ; Kligler and Olitzki, 1932) have denied that this is possible unless the supposed neutral mixture contains some unneutralized phage. We agree with their criticisms so far as the ordinary methods of demonstrating neutralization are concerned. If a few drops of a *B. coli* culture are seeded into a test-tube containing equal parts of freshly mixed C36 and undiluted antiserum, no lysis will occur ; the phage will progressively diminish and disappear. Diluted antiserum is ineffective. If

the "neutral" mixture is diluted 1 : 10 or up to 1 : 100,000 before adding the *B. coli*, phage multiplication and lysis will take place; reactivation of the neutral mixture has apparently occurred. But it can readily be shown that undiluted serum will prevent the progressive multiplication of phage even if this has had an opportunity of infecting the organisms before the serum is added; dilutions of serum of 1 : 10 or more cannot produce this inhibiting effect. One of us has discussed possible explanations of these facts in an earlier paper (Andrewes, 1932). It is clear, then, that an apparently neutral mixture of phage and undiluted serum may contain free phage, and yet fail to produce lysis; dilute it so that the serum is beyond the range in which it can inhibit the lysis and there will still be plenty—even 100,000 per c.c. —free phage particles left; these can proceed to multiply. Clearly, experiments on these lines cannot prove that there is any dissociation by dilution of a phage-serum complex. The experiments of Prausnitz (1923) and of Weiss (1927) purporting to show dissociation of neutral mixtures by heat and trypsinization respectively may be vitiated by the same fallacies.

REACTIVATION OF INCOMPLETELY-NEUTRALIZED PHAGE.

Nevertheless, we believe that in another way we *have* demonstrated that dilution may cause dissociation of a phage-antibody complex. A mixture of equal parts of phage C36 and 1 : 10 antiserum was left 2 hours at 37° and then diluted 1 : 1000 in broth. 0·1 c.c. quantities of 1 : 10 and 1 : 100 dilutions of this were spread on agar plates at once, and again after standing for 4 hours at 20° C.

TABLE III.—*Dissociation of Diluted Phage Serum Mixture on Standing.*

	Tested at once.	Tested after 4 hours.
Numbers of plaques { dilution 1 : 10	19	217
Numbers of plaques { ,, 1 : 100	1	34
Average size of 20 plaques	0·20 mm.	0·40 mm.

Table III shows that after standing, the titre was apparently increased more than tenfold. Further, the average plaque size was doubled. This experiment was repeated many times and, with this batch of antiserum, always with success. With another, more potent, anti-C36 serum the effect was not demonstrable. With the first lot of serum the following facts were elicited. There was very little increase in titre to be shown within an hour, but after standing for 4 or 5 hours the dissociation had practically reached its maximum. The phenomenon was shown slightly better at 37° than at 20°, and definitely better at 20° than at 0° C. The amount of dilution beyond 1 : 100 did not affect the result. Dissociation was no better in a drop spread on the surface of an agar plate than in broth. It was unaffected by the presence of killed *B. coli* at 37°, of living *B. coli* at 0° C., or of living *B. coli* multiplying at 37° in the presence of 1% sodium citrate (this dilution of citrate prevents multiplication of C36). We could not, of course, test the effect of the presence

of multiplying *B. coli* without citrate, for the multiplication of phage would have obscured the result. Although the average plaque size increased when mixtures were left standing, as in the experiment in Table III, from 0·2 to 0·4 mm., indefinitely prolonged standing did not bring the size up to the normal (about 0·7 mm.).

We could not find any evidence of dissociation of a mixture which had been incubated for 4 hours at 37° before diluting. Nor could we elicit the phenomenon at all with phages C13 and C16. Probably with certain phages and certain sera the reaction goes fairly slowly through a stage in which dissociation is possible; with other combinations the steps may follow each other so rapidly that this intermediate stage cannot be studied. Burnet (1933, *a*) has also obtained some evidence pointing to reversal of phage-antibody union in the presence of his "soluble substance derived from phage".

ACTION OF INCOMPLETELY-NEUTRALIZED PHAGE (C36) ON B. COLI.

At first sight these experiments appeared to afford a satisfactory explanation of the delay before the beginning of multiplication of phage when diluted phage-serum mixtures were incubated with *B. coli*. We supposed that phage in such mixtures was gradually dissociated by the dilution from its union with antibody, and that only then could it attack the organisms. But after incubation for 4 hours (after which time dilution produces no more reactivation) —or even for days—phage in such a mixture still only begins multiplying after a delay, and produces only small plaques on agar. The condition of "incomplete neutralization" is thus a permanent one depending probably on the amount of antibody attached to the phage, and not to be confused with the temporary condition of the phage-serum complex in which the neutralization is reversible. We can, if we like, suppose that in the incompletely neutralized phage the phage-antibody union can be broken only in the presence of living multiplying *B. coli*; but it is hard to understand the reason for this, since we have already mentioned that *B. coli* growing in the presence of citrate do not assist reactivation. Possibly the coating of antibody is shed only as the phage enters—with difficulty and delay—a susceptible bacterium. But in our present state of ignorance of the intimate mechanism of the infection of bacteria by phage, further speculation is useless.

DISCUSSION.

We will now put forward very tentatively a working hypothesis which seems to us to cover reasonably well the facts we have recorded. Phage-neutralization may be supposed, in common with other antigen-antibody reactions, to consist of two stages. The first stage probably takes place very rapidly, for certain phage-serum mixtures become non-filterable immediately on mixing. The first stage may also be reversible, since dilution can, for a time, produce reactivation of a mixture of C36 and antiserum. The second stage is, so far as we have been able to show, irreversible; it probably follows gradually upon the initial reversible phase in the case of C36, but more rapidly in reactions with anti-C16 and anti-C13 sera.

We do not know what are the physical differences which bring about the different behaviour of " incompletely " and the " completely neutralized " phage. The former apparently has its physical properties changed, and is able to attack the sensitive bacterium only after a delay. If we may judge from plaque-size (*cf.* Table I), the more antibody is attached to the phage, the greater will be the delay. It seems possible that phage has a number of centres which have affinity for the bacterium, and that it is completely neutralized only when all these are masked by the antibody.

There is much evidence suggesting that the surface of the phage has active centres of two kinds—one with affinity for antibody (A), the other with affinity for the bacterium (B). (A) determines the serological specificity of the phage, (B) the range of bacterial species which it can attack. Burnet (1932) has shown that these affinities may vary independently. He has suggested to us that the neutralization of the antigenic (A) centres by antibody may produce the condition of partial neutralization ; the adsorption of additional antibody on to these neutralized centres may lead to the progressive blanketing of the adjacent (B) centres ; only when these are covered up will the phage be truly neutralized. Such an explanation would account for the need for excess antibody to ensure neutralization, and for the existence of a partly-neutralized phage with definite properties. But it is, however attractive, a theory of which the truth is hard to prove.

We are now in a better position to discuss the meaning of the " percentage law " described in the previous paper. If the affinity of phage of antibody is low, we may believe that the equilibrium is of such a nature that the phage cannot receive a big enough coating of antibody to neutralize it, unless there is a considerable excess of antibody in the fluid around each phage particle. If this excess is always necessary, it follows that, over the whole range under study, phage particles must be few and far between compared with the available antibody. We can, therefore, best study what happens in the mixture by concentrating on the fate of each individual phage particle in its sea of antiserum ; what happens to the other phage particles will not affect its destiny at all. As we pointed out earlier, the amount of antibody in the fluid around each phage particle will doubtless determine how much becomes attached, and this in turn will decide whether or not the phage is neutralized. The resistance, of a certain percentage of phage to each serum-dilution may depend upon innate differences in serum-resistance of the individual particles, or less probably upon accidental differences in the molecular arrangements of the phage-antibody system.

Schultz (1928) has urged that reactions between viruses, including phage, and antibodies resemble toxin-antitoxin unions rather than those by means of which the body deals with bacterial bodies. We do not believe that the distinction is a fundamental one, but in any case our experiments suggest that, so far as phage is concerned, the analogy with toxins and antitoxins is by no means close. Phage is intermediate in size between toxins and bacteria, and peculiarities in the mechanism of its inactivation by antiserum may depend upon that fact.

Further work will be necessary to show whether antiviral sera behave like antiphage sera in the ways discussed in this paper. Differences we have

found between the action of one antiphage serum and another will suffice to warn us against too sweeping generalizations.

CONCLUSIONS.

1. Phage which is incompletely neutralized by—perhaps incompletely coated with—antiserum, when it is brought into contact with susceptible bacteria, only begins to multiply after a delay. For this reason it produces smaller plaques on agar than does free phage (C36, not C13 and C16).

2. This incompletely neutralized phage is normally adsorbed by susceptible bacteria (C36).

3. This incompletely neutralized phage is unable to pass a candle or a membrane permeable to phage and antiserum separately. Probably there is aggregation of the phage-serum complex (C36, S18).

4. Phage which has been incompletely neutralized or even completely coated by antiserum may be reactivated by dilution, provided that there has not been too long contact of phage and antibody (C36, not C13, C16).

5. A working hypothesis to cover the above facts has been put forward.

During the later part of our work we have had the advantage of much helpful discussion with Dr. F. M. Burnet.

REFERENCES.

ANDREWES, C. H.—(1932) *Brit. J. Exp. Path.*, **13**, 85.
BURNET, F. M.—(1929) *Ibid.*, **10**, 109.—(1933, *a*) *Ibid.*, **14**, 100.—(1933, *b*) *Ibid.*, **14**, 302.
ELFORD, W. J.—(1931) *J. Path. Bact.*, **34**, 505.
D'HÉRELLE, F.—(1921) ' Le bactériophage : son rôle dans l'immunité '. p. 109. Paris (Masson et Cie).
KLIGLER, I. J., AND OLITZKI, L.—(1932) *Brit. J. Exp. Path.*, **13**, 237.
MUCKENFUSS, R. S.—(1928) *J. Exp. Med.*, **48**, 709.
OTTO, R., AND MUNTER, H.—(1922) *Z. Hyg. InfektKr.*, **98**, 302.
PRAUSNITZ, C.—(1923) *Zbl. Bakt.*, I. O., **80**, 187*.
SCHLESINGER, M.—(1933) *Z. Hyg. InfektKr.*, **114**, 746.
SCHULTZ, E. W.—(1928) *J. Immunol.*, **15**, 229.
Idem, QUIGLEY, J. S., AND WOOLSEY, E. L.—(1931) *Ibid.*, **20**, 149.
SEIFFERT, W.—(1923–24) *Z. ImmunForsch.*, **38**, 292.
WEISS, E.—(1927) *J. Immunol.*, **13**, 301.

THE EFFECT OF HYPOPHYSECTOMY ON THE GROWTH OF THE WALKER RAT TUMOUR.

C. S. McEUEN AND D. L. THOMSON.

From the Department of Biochemistry, McGill University, Montreal.

Forwarded for publication August 25th, 1933.

SINCE hypophysectomy is known to lead to cessation of somatic growth, except in very young animals, its effect upon the growth of tumours becomes a question of interest. The experiments described in this paper deal with the effects of hypophysectomy upon the growth of the Walker rat tumour in albino rats of Wistar strain. This tumour was found by Dr. George Walker, Baltimore, in the breast of a female rat in 1928, and has been regarded as a carcinosarcoma. It was made available to us through the kindness of the Rockefeller Institute for Medical Research, and has since been carried in this laboratory. In our hands it gives 76 per cent. of takes, rarely regresses after 14 days, and may attain a size of more than 100 gm. The animals used in these experiments were almost without exception males, 60 to 80 days old; each received a single transplant, in the left flank, from tumours 10 to 12 days old.

HYPOPHYSECTOMY AFTER TRANSPLANTATION.

A preliminary report of the first series of experiments has already been published (McEuen, 1933); the results are summarized in Table I. Twenty-six rats bearing tumours 10 to 12 days old were matched in pairs, so that the members of each pair had tumours of similar age and size. One member of each pair was then hypophysectomized by a parapharyngeal operation (Collip, Selye and Thomson, 1933); one animal was lost. Calliper measurements of one

TABLE I.—*First Series.*

Days after operation.	Hypophysectomized. (Average of 12 rats.)		Controls. (Average of 13 rats.)	
	Tumour area (cm.²).	Total weight (gm.).	Tumour area (cm.²).	Total weight (gm.).
0	3·8	176	3·3	188
12	8·2	170	10·9	203
18	10·7	171	15·4	216
24	13·1	..	19·3	..

plane of the tumours were recorded, and the total weights were followed. As the hypophysectomized rats died off, 21 to 36 days after operation, the corresponding controls were sacrificed. It was found that the tumours increased in size in the hypophysectomized rats more slowly than in the controls; the

total weights of the controls increased progressively, while those of the hypophysectomized animals remained stationary or declined somewhat, especially in the last week before death.

These results appeared to justify further investigation. A second series of experiments was therefore carried out on a similar plan, save that in this case the animals were examined *post mortem* for the completeness of hypophysectomy and the presence of metastases, and the tumours were dissected out and weighed (with the overlying skin, but without metastases). The records of the eight operated animals of this series (one incomplete hypophysectomy) are arranged in Table II in order of length of survival after operation, with the data for the corresponding controls below. The change in total weight is

TABLE II.—*Second Series.*

Hypophysectomized :	Rat No. 3PB.	4GH.	3YH.	3PT.	3YT.	4YT.	4YH.	4VB.
Age of transplant at operation (days)	14	25	22	14	18	13	13	22
Age of transplant at death (days) .	19	33	31	30	36	31	34	47*
Gain or loss in total weight (gm.) .	−10	−15	−16	−2	+13	−2	−11	−21
Tumour weight (gm.) . . .	10	39	16	33	25	37	21	40
Metastases	?	0	?	0	0	0	0	R

Controls :	Rat No. 3PT.	4PT.	3YB.	3VH.	3PH.	4PH.	4YB.	4VH.
Gain or loss in total weight (gm.) .	+7	−6	+10	+34	+54	+29	+12	+5
Tumour weight (gm.) . . .	16	51	23	44	54	70	61	53
Metastases	?	R	?	R	P	0	R	M.P.R

* Incomplete hypophysectomy ; R, retroperitoneal ; P, pulmonary ; M, mediastinal.

recorded for the period between hypophysectomy and death, or for the corresponding length of time and tumour age in the controls. It will be noted that in every case the tumours carried by the hypophysectomized rats at death were smaller than those of the corresponding controls sacrificed at the same length of time after inoculation. Since the early mortality in these hypophysectomized rats is very much greater than in untreated hypophysectomized rats without tumours in this colony, it appears that hypophysectomy makes the animals more apt to succumb to the effects of the developing tumour.

TRANSPLANTATION AFTER HYPOPHYSECTOMY.

In the third series of experiments, the transplantations were made into rats which had been hypophysectomized 7 to 19 days earlier ; within this range the lapse of time after operation does not appear to be important. Samuels, Ball and Simpson (1933), in a similar experiment, apparently with this same tumour, allowed a longer interval between operation and inoculation, and administered small doses of thyroid substance to their animals. They obtained only 4 takes from 68 transplants, while 38 per cent. of their transplants into normal animals were successful ; analysis of these figures by the χ^2 test shows that there is hardly one chance in a million of this result being due merely to error of sampling. In the present work, however, this effect did not appear ;

the proportion of unsuccessful transplants in the hypophysectomized animals, though greater than in the control experiments reported in this paper, is not greater than in normal rats in this laboratory in general; both before and after the experiments herein described, the percentage of takes with this tumour in this colony was found to be approximately 76. Since the present experiments fully confirm those of Ball, Samuels and Simpson (1932) in other respects, notably in the retardation of tumour growth after hypophysectomy, this discrepancy in the percentage of takes, which again is quite beyond the range of sampling errors, is peculiarly interesting.

TABLE III.—*Third Series.*

Rat.	Survival (days)— after transplant.	Survival (days)— after operation.	Gain or loss in total weight (gm.).	Tumour weight (gm.).	Metastases.
6BT	6	18	− 9	Reg.	?
5VB	24	31	+ 8	L	?
7PB	25	44	− 2	5	0
7VH	26	45	+ 3	10	0
6YB	28	42	− 18	Reg.	0
5YT	29	36	− 7	12	R
5PH	29	36	− 12	10	0
5YH	30	37	− 11	19	0
5YB	30	37	+ 29	31	0
5PB	33	40	+ 7	10	0
6BH	36	48	0	18	R
7YH	37	56	+ 15	19	0
7YT	39	58	+ 9	21	0
6PH	40	54	+ 28	30	0
6PT	53K	67	+ 29	Reg.	0

Reg., tumour regressed; L, lost; K, killed; R, retroperitoneal.

The third experimental series also indicates that tumours transplanted into hypophysectomized rats grow abnormally slowly and rarely give rise to metastases, in spite of which the inoculated hypophysectomized rats, with one exception, died within six weeks. Some of the animals in this series showed considerable gains in total weight, although only in one instance (in which regression occurred) did this gain exceed the weight of the tumour. There is no obvious explanation for this behaviour, as in none of the cases did *post-mortem* examination suggest that the hypophysectomy was incomplete. The results are presented in Table III; the change in total weight recorded refers to the period between inoculation and death.

THE EFFECT OF PARTIAL STARVATION.

The fourth experimental series was designed partly as a control for the third, to indicate the size which tumours might be expected to attain in normal animals in similar time-intervals, but was also planned to shed light on a

question which the earlier work had seemed to raise. It appeared to be important to know whether the retarded tumour growth in the hypophysectomized rats was a specific result of the operation, or whether retardation of growth is regularly accompanied by retarded tumour growth. Bischoff and Maxwell (1931) showed that various drugs which impaired growth in general did not check the growth of the Hyde rat carcinoma and the rat sarcoma 10, and elsewhere (Bischoff, Maxwell and Ullmann, 1931) they state that this is true also of starvation. Rous (1911, 1914) found that underfeeding checked

TABLE IV.—*Fourth Series.*

Age of transplant (days).		Restricted diet.				Unrestricted diet.				Control for Series III rat.
Initial.	Final.	Rat.	Total weight gain.	Tumour weight (gm.).	Metastases.	Rat.	Total weight gain.	Tumour weight (gm.).	Metastases.	
11	22	09YH	−12	6	0	78YH	+19	10	0	5VB
12	25	79YT	−6	7	0	75YT	+20	9	0	7PB
14	26	52YH	−5	22	0	66YH	+35	23	0	7VH
13	26	10YH	−13	52	0	38YH	+25	56	R	..
13	26	45YB	−13	63	R	38YB	+24	65	R	..
14	28	51PT	−7	32	R	66PT	+34	37	0	6YB
11	29	27YB	−5	21	0	75YB	+36	16	0	5YT
11	29	48	−2	17	0	66PH	+48	17	0	5PH
17	30	04PH	−4	25	R	81PH	+31	26	0	5YH
10	30	17	−9	22	0	61YH	+26	30	R	5YB
8	30	62	−2	49	R	61YT	+29	Reg.	0	..
16	33	25PB	+3	38	R	81PB	+41	46	0	5PB
12	36	07VH	−10	40	R	78VH	+36	42	R	6BH
9	37	36	+2	13	0	35YH	+36	18	R	7YH
15	39	46	0	28	0	67PH	+41	45	0	7YT
9	40	80	+1	35	R	67PT	+64	64	R	6PH
10	42	51YH	−20	57	R	78PH	+13	58	R	..
11	43	52BH	−19	53	R	35BH	+43	70	R	..
14	44	73VH	−11	45	0	35VH	+32	73	R	..
8	46	82	−12	94	0	35YB	D	..	..	..
11	47	81VB	−15	59	R	66VB	+50	78	R	..
14	50	25VH	−27	60	0	67VH	+45	64	R	..
15	53	00	−8	64	0	61PH	+45	108	R, P	6PT

Reg., tumour regressed; D, died; R, retroperitoneal; P, pulmonary.

the growth of the Jensen rat sarcoma but not that of the Flexner-Jobling rat carcinoma. These findings, and those of earlier workers quoted by Rous (1914), suggested that the problem should be investigated for the same tumour and under the same conditions as in our previous experiments. Accordingly 46 rats were matched in pairs of equal tumour size and age, as before; one member of each pair received the stock diet *ad lib.*, while the other received water *ad lib.*, but only enough of the same diet to keep the total weight constant or slightly decreasing. The rats were weighed and the tumours measured every second day, and the pairs were sacrificed at various intervals (partly so as to correspond in length of tumour life to the hypophysectomized rats of Series III) and examined *post mortem* as before.

The results are summarized in Table IV, which records the age of the transplants at the beginning of the period of observation (when restriction of

diet for half the rats was commenced) and at the time of killing, as well as the change in total weight over this period and the tumour weight and occurrence of metastases at death. There can be no doubt that semi-starvation does retard the growth of this tumour. The average difference in weight of tumour between each semi-starved animal and its control is 9 gm., and it may be shown by the use of the statistic t (Fisher, 1932) that the odds against this being a random difference greatly exceed 100 to 1. It is true that in the fully-fed group there appears to be no correlation between the rate of increase of the total weight of the animals and the rate of growth or the final size of the tumours, nor can the growth of the tumours be correlated with the initial weight of the recipient. It cannot, however, be affirmed that such relationships do not exist, since the ranges of the variables in these experiments are small, and since the experiment was planned to facilitate comparison between the members of each pair rather than between the members of each group.

DISCUSSION.

Occurrence of Metastases.

Metastases were seldom observed in hypophysectomized rats ; but in these experiments they were also rare in those normal rats whose primary tumours weighed less than 30 gm. If one considers only the cases in which the primary tumour weighed from 12 gm. (the smallest weight associated with the presence of a metastasis) to 40 gm. (the largest weight attained by a tumour in a hypophysectomized animal), the tendency for metastases to occur more frequently in normal than in hypophysectomized animals, tested by χ^2, is not statistically significant. Hence it cannot be affirmed that hypophysectomy specifically inhibits the formation of metastases.

Decrease of Net Weight.

Attention was drawn in the preliminary note (McEuen, 1933) to the behaviour of the " net " weights (total weight *minus* tumour weight) of the animals ; it was suggested that the net weights decreased at approximately the same rate in normal and in hypophysectomized rats. This suggestion was based merely upon estimations of the tumour weights, and it was therefore important to check it in the later experiments where the tumour weights were accurately known. As individual variations in net weight change are very great, it was necessary to study the average behaviour statistically. In series II, the average decline of the net weight in the hypophysectomized rats (excluding the one incompletely hypophysectomized) was 32 gm., and 27 gm. in the control animals. It may be shown by the use of t that the odds are slightly in favour of the view that this apparent difference is due to sampling ; moreover, the difference would undoubtedly have been less had the weights been determined a few days before the death of the hypophysectomized animals, and is also partly due to the inclusion of the weight of the metastases, small though they are, in the net weight. There is still greater variation in the net weight losses of the fully-fed rats of Series IV ; it averages 11·5 gm. (which happens to be almost exactly equal to the average net weight loss of the hypophysectomized animals of Series III), but there is one chance in twenty that

the apparent loss is fictitious, and would disappear in a sufficiently large series. There is less than one chance in a hundred that this is true of the Series II controls, and though these last appear to show much greater net weight loss than the apparently comparable animals of Series IV, the difference is not statistically significant, the odds in favour of its reality (tested by *t*) being less than 9 to 1. It may be concluded that the net weight of control tumour-bearing rats does tend to decline, at a rate which is not significantly less than in hypophysectomized animals. It must be recalled that this statement applies to averages and not to individuals.

Tumour Weight and Net Weight Loss.

It was further suggested in the preliminary note that the tumour weight in hypophysectomized rats was equal to the net weight loss ; in other words, that the total weight remained constant. The data recorded in Table II indicate that the total weight usually decreased ; but, as we have already observed, this decrease usually occurs in the last few days of life, when the animals are probably in advanced cachexia. The original protocols show that in most instances the hypophysectomized tumour-bearing rats of Series I and II maintained their weight at the time of operation within 4 or 5 gm. *plus* or *minus*, until this terminal decline set in ; in the ten rats from these series which survived hypophysectomy by 3 weeks or more, the average weight change from the fifth to the fifteenth day after operation was an increase of only 0·73 per cent. (standard deviation $\pm$ 2·77). In this respect the animals resemble hypophysectomized rats without tumours. It may therefore be said that in the hypophysectomized rats the tumour grows solely at the expense of the existing normal tissues. This statement is a description, not an interpretation ; we do not mean to imply that all or any of the molecules which constitute the neoplasm were previously located in the protoplasm of the normal tissues, since we know of no evidence on this question.

In the control rats with unrestricted food supply, the tumour weight in general greatly exceeds the loss in net weight. For 27 animals from Series II and IV, the average net weight loss was 16·15 gm. (standard deviation $\pm$ 24·62, indicating that one animal in four may be expected to show increasing net weight) and the average tumour weight was 47·22 gm. (standard deviation $\pm$ 22·65) ; the average weight of metastases, were it known, should be added to *both* these figures. It may therefore be said that in these control animals the tumours grew partly at the expense of the existing healthy tissues and partly by retention of assimilated food-stuffs. As the drain upon the normal tissues was not significantly less than in the hypophysectomized animals, the growth of the tumours was more rapid in the controls. As might be anticipated, large tumours are generally associated with large net weight losses, the correlation coefficient (0·791) exceeding ten times its standard deviation.

Restriction of Diet Compared with Hypophysectomy.

It now becomes interesting to compare the hypophysectomized rats and the partially starved rats with respect to rate of net weight loss and of tumour growth, since in both groups tumour growth occurs solely at the expense of

normal tissue and the total weight is approximately constant. We propose to devote more experiments to this comparison; the data already collected afford some information, but the interpretation is not clear. It is possible, in the first place, to match the tumours of the hypophysectomized rats (Series I and II) for size and age against similar tumours borne by Series IV rats at the beginning of the period of diet restriction, and to follow the subsequent development of these pairs of tumours from the recorded calliper measurements and sketches; this comparison reveals no difference between hypophysectomized rats and starved rats. It is possible, secondly, to compare the hypophysectomized rats of Series II with those of the starved rats which were killed within 36 days of inoculation; on the average, the latter display heavier tumours and greater net weight loss than the former, but the difference is not statistically significant. If a real difference exists, it is necessary to suppose that hypophysectomy not only prevents the addition of new material to the system, but also retards the apparent transfer of material from the normal tissues to the tumour; our data, however, do not justify this additional hypothesis. They are adequately explained by the suppositions that hypophysectomy prevents the retention of food-stuffs and increase of total weight, and that it also lowers the resistance of the animals, so that death occurs before the tumours have attained large size. It may be observed that the net weight loss of the starved animals is significantly greater than that of the fully-fed controls, which indicates that the healthy tissues are to some extent spared in the presence of an abundant food supply.

It is beyond the scope of this paper to discuss the manner in which hypophysectomy prevents increase of total weight. In this laboratory, experiments on hypophysectomized rats without tumours have shown that their intake of food is abnormally small, but their nitrogen excretion is abnormally high; we are, therefore, disinclined to attribute the constancy of weight wholly to loss of appetite. The mechanism of action of the so-called growth hormone is obscure. Moreover, hypophysectomy affects the function of other endocrine glands and causes primary and secondary metabolic disturbances, and we are not justified in assuming that its effect upon tumour growth is wholly due to withdrawal of the growth hormone. Two lines of research which may possibly provide an answer to this question are being followed. In the first place, the effect of various partly purified hypophysial extracts on the growth of this tumour is being investigated; and secondly, a study is being made of the effect of tumour growth upon various endocrine glands in normal and hypophysectomized animals.

SUMMARY.

The growth of the Walker rat tumour in young male albino rats is retarded by hypophysectomy, and the resistance of the animals is lowered. Metastases occur less frequently in hypophysectomized rats, probably because large tumours are rare. Inoculation into hypophysectomized rats is successful in a large proportion of cases, but the growth of the transplants is slow.

The total weight of the tumour-bearing rats remains constant after hypophysectomy, but increases in the controls. If this increase is prevented by

restricting the intake of food, the growth of the tumours is retarded. This retardation is not significantly less than that produced by hypophysectomy.

The body-weights of the rats *minus* their tumours tend to decrease; this decrease is approximately equal to the weight of the tumour in hypophysectomized rats, but not in normal rats.

We desire to express our grateful thanks to Prof. J. B. Collip for his interest in this research, to Dr. Hans Selye for performing the hypophysectomies, and to Miss Silby Harrison and Miss J. E. Williamson for technical assistance.

REFERENCES.

Ball, H. A., Samuels, L. T., and Simpson, W.—(1932) *Am. J. Cancer*, **16**, 351.
Bischoff, F., and Maxwell, L. C.—(1931) *J. Pharmacol.*, **42**, 387.
Idem, and Ullmann, H. G.—(1931) *Science*, **74**, 16.
Collip, J. B., Selye, H., and Thomson, D. L.—(1933) *Arch. path. Anat.*, in press.
Fisher, R. A.—(1932) 'Statistical Methods for Research Workers,' 4th edit., Edinburgh (Oliver & Boyd), p. 112.
McEuen, C. S.—(1933) *Proc. Soc. Exp. Biol. Med.*, **30**, 928.
Rous, P.—(1911) *Ibid.*, **8**, 128; (1914) *J. Exp. Med.*, **20**, 433.
Samuels, L. T., Ball, H. A., and Simpson, W.—(1933) *Am. J. Cancer*, **18**, 380.

EXPERIMENTS ON THE FILTRATION OF YELLOW FEVER VIRUS THROUGH "GRADOCOL" MEMBRANES.

G. M. FINDLAY and J. C. BROOM.

From the Wellcome Bureau of Scientific Research, London.

Received for publication September 6th, 1933.

The majority of the experiments reported below have been carried out with a French strain of yellow fever virus which had been fixed for mice by repeated passage in mice brains as first described by Theiler (1930). For this strain we are indebted to Dr. W. A. Sawyer, Director of the Yellow Fever Laboratories of the International Health Division of the Rockefeller Foundation, New York. After 20 to 30 passages in mice brains the strain loses its viscerotropic qualities and, on subcutaneous or intraperitoneal inoculation into *rhesus* monkeys, shows no tendency to produce typical yellow fever; inoculated intracerebrally, or intraperitoneally with simultaneous damage to the brain, it causes a fatal encephalitis in monkeys as in mice.

This strain has been used by Sawyer, Kitchen and Lloyd (1932) and by Findlay (1933) for human prophylactic immunization.

Material.

The strain of neurotropic yellow fever virus employed had undergone from 230 to 310 passages in mice brains. Infected mice were killed when they

showed marked symptoms of encephalitis, usually on the fourth day after inoculation, and the brains, removed aseptically, were ground with enough of a 1/10 dilution of normal human serum in saline to produce a 1/5 or 1/10

TABLE I.—*Filtration of Neurotropic Strain of Yellow Fever Virus.*

Average pore-size of membrane.	Thickness.	Filtration pressure.	Volume filtered, c.c. per sq. cm.	Infectivity of filtrate (0·03 c.c. intracerebrally).	Infectivity of stock mouse brain suspension (0·03 c.e. intracerebrally).
0·61μ	0·16 mm.	20 cm. Hg.	3	10^{-4}	10^{-4}
0·5μ	0·16 ,,	20 ,,	3	10^{-5}	10^{-5}
0·36μ	0·20 ,,	20 ,,	3	10^{-4}	10^{-4}
0·25μ	0·17 ,,	20 ,,	5	10^{-5}	10^{-5}
0·25μ	0·17 ,,	40 ,,	5	10^{-5}	10^{-5}
0·15μ	0·30 ,,	20 ,,	5	10^{-3}	10^{-5}
0·15μ	0·16 ,,	40 ,,	5	10^{-4}	10^{-5}
0·15μ	0·16 ,,	40 ,,	5	10^{-5}	10^{-5}
90μμ	0·30 ,,	40 ,,	5	10^{-3}	10^{-5}
90μμ	0·30 ,,	40 ,,	5	10^{-2}	10^{-3}
80μμ	0·16 ,,	40 ,,	5	Undiluted; 4 of 6 mice dead.	Undiluted;* 6 of 6 mice dead.
80μμ	0·16 ,,	40 ,,	5	Undiluted; 9 of 12 mice dead.	Undiluted;* 6 of 6 mice dead.
70μμ	0·30 ,,	40 ,,	10	Undiluted; 2 of 6 mice dead.	Undiluted;* 6 of 6 mice dead.
70μμ	0·30 ,,	40 ,,	10	Undiluted; 3 of 6 mice dead.	Undiluted;* 6 of 6 mice dead.
55μμ	0·20 ,,	40 ,,	10	Undiluted;† 1 of 6 mice dead.	Undiluted;* 6 of 6 mice dead.
55μμ	0·20 ,,	60 ,,	20	Undiluted;‡ 0 of 6 mice dead.	Undiluted;* 6 of 6 mice dead.
55μμ	0·20 ,,	60 ,,	30	Undiluted; 0 of 6 mice dead.	Undiluted; 6 of 6 mice dead.
50μμ	0·25 ,,	40 ,,	10	Undiluted; 0 of 12 mice dead.	Undiluted;* 6 of 6 mice dead.
50μμ	0·18 ,,	60 ,,	20	Undiluted; 0 of 12 mice dead.	Undiluted;* 6 of 6 mice dead.
50μμ	0·18 ,,	2 atmos.	20	Undiluted; 0 of 12 mice dead.	Undiluted;* 6 of 6 mice dead.

* Not tested to titre.
† Possibly non-specific death.
‡ Suspension of guinea-pig brain, sixteenth passage.

TABLE II.—*Concentration of Virus Above a 50μμ Membrane.*

Average pore-size of membrane.	Thickness.	Filtration pressure.	Test of filtrates.	Test of final supernatant after 5 times dilution (0·03 c.c. intracerebrally).	Infectivity of stock suspension (0.03 c.c. intracerebrally).	Relative concentration.
50μμ	0·2 mm.	40 cm. Hg.	Undiluted 0	10^{-8}	10^{-4}	$\times 10^4$
50μμ	0·2 ,,	40 ,,	,,	10^{-8}	10^{-5}	$\times 10^3$

emulsion. The emulsion was centrifuged for 15 minutes at 4000 revs. per min. and the supernatant fluid filtered through a sand-and-pulp filter (Barnard and Elford, 1931). The filtrate was passed through a gradocol membrane of A.P.S. 0·7–0·8 μ to give a bacteria-free stock suspension for use in the filtration tests.

TABLE III.—*Filtration of Viscerotropic Yellow Fever Virus (French Strain).*

Date.	Average pore-size of membrane.	Thickness.	Filtration pressure.	Volume filtered, c.c. per sq. cm.	Number of days till death.	Histological examination of liver.
25.vii.33	0·1μ	0·24 mm.	40 cm. Hg.	5	8	+
1.viii.33	0·1μ	0·24 ,,	40 ,,	5	6	+
25.vii.33	87$\mu\mu$	0·20 ,,	40 ,,	5	8	+
1.viii.33	87$\mu\mu$	0·20 ,,	40 ,,	5	9	+
25.vii.33	75$\mu\mu$	0·23 ,,	40 ,,	5	10	+
1.viii.33	75$\mu\mu$	0·23 ,,	40 ,,	5	11	+
25.vii.33	55$\mu\mu$	0·20 ,,	40 ,,	5	11	+
1.viii.33	55$\mu\mu$	0·20 ,,	40 ,,	5	Survived.	—
25.vii.33	Control	..	..	..	6	+
1.viii.33	,,	..	..	..	5	+
25.vii.33	Supernatant of 55$\mu\mu$ remaining after filtration.	..	..	..	5	+

Technique.

As in our experiments on the filtration of Rift Valley fever virus (Broom and Findlay, 1933), we have followed the methods described by Elford (1931, 1933) for the preparation and use of collodion filters of regular and graded pore-size. Unless otherwise stated, each dilution of test material was inoculated intracerebrally into six mice. Table I shows the results of filtration experiments through a series of membranes of decreasing pore-size.

The first drop in titre of the filtrate as compared with the stock suspension would appear to occur between 0·15 μ and 0·1 μ A.P.S., while the limiting pore-size is 55 $\mu\mu$.

Elford (1933) has pointed out that the pore-size of the membrane which causes the first fall in titre is usually approximately twice that of the limiting membrane. Since this grade of membrane (55 $\mu\mu$) retains particles of one-half to one-third the size of the pore, the diameter of the virus particles should be between 18 and 27 $\mu\mu$.

Concentration Experiments.

Relative concentration of the virus above a membrane through which it could not pass was obtained in the following way: 20 c.c. of a 1/10 suspension in serum saline was filtered through a 50 $\mu\mu$ membrane until 19 c.c. had passed; 9 c.c. of serum saline was added to the 1 c.c. remaining above the membrane and filtration continued. This was repeated a further four times, so that the final relative concentration of the virus above the membrane was 2×10^{-5} of the original suspension. The final supernatant fluid and the original stock suspension were tested to titre, and each 9 c.c. filtrate was also tested undiluted for the presence of virus. Table II shows that under these conditions relative concentration can be obtained above a 50 $\mu\mu$ membrane.

Viscerotropic Virus (French Strain).

To test whether these measurements of the size of the virus particles might be applied to the normal viscerotropic virus, two small series of filtrations were carried out with a suspension prepared from monkeys dead of typical

yellow fever. Liver was used as the source of the virus, but otherwise the technique was the same as that employed for the neurotropic virus. Only one monkey was inoculated with the undiluted filtrate in each experiment.

Table III gives the results of these experiments, from which it appears probable that the viscerotropic virus is within the same range of size as the neurotropic strain. It is of interest that Elford, Perdrau and Smith (1933) found that cerebral and testicular strains of herpes virus were also of the same size.

SUMMARY AND CONCLUSIONS.

1. By the use of "Gradocol" membranes the size of a neurotropic strain of yellow fever virus is estimated to lie between 17 and 28 $\mu\mu$.

2. The relative concentration of the virus has been obtained above membranes impermeable to it.

3. From a few experiments it appears that the viscerotropic yellow fever virus is probably of the same size as the fixed neurotropic strain.

REFERENCES.

BARNARD, J. E., AND ELFORD, W. J.—(1931) *Proc. Roy. Soc.*, B, **190**, 363.
BROOM, J. C., AND FINDLAY, G. M.—(1933) *Brit. J. Exp. Path.*, **14**, 179.
ELFORD, W. J.—(1931) *J. Path. Bact.*, **34**, 505.—(1933) *Proc. Roy. Soc.*, B, **112**, 384.
Idem, PERDRAU, J. R., AND SMITH, W.—(1933) *J. Path. Bact.*, **36**, 49.
FINDLAY, G. M.—(1933) *Bull. Office Internat. Hyg. Publ.*, **25**, 1009.
SAWYER, W. A., KITCHEN, S. F., AND LLOYD, W.—(1932) *J. Exp. Med.*, **55**, 945.
THEILER, M.—(1930) *Ann. Trop. Med.*, **24**, 249.

THE REACTION OF THE ARTERIAL BLOOD IN CANCER.

SYLVIA DICKINSON AND R. E. HAVARD.

From the Departments of Physiology and Experimental Pathology, The University of Leeds.

Received for publication September 21st, 1933.

A CONSIDERABLE amount of work has been done on the hydrogen-ion concentration of the blood of subjects suffering from cancer, but the findings of independent workers have not all been in agreement.

Some investigators have found that the blood of patients with cancer is more alkaline than that of normal subjects (Chambers, 1923; Bischoff, Long and Hill, 1930; Reding, 1928; Reding and Slosse, 1930, *a* and *b*, Hueper, Woodward and Fry, 1931; Woodward and others, 1930–31; McDonald and Hueper, 1930–31). Of these workers, some state that the increased alkalinity occurs only in cases of "internal cancer" and not in

cases of "surface cancer" (McDonald, 1927; Woodward and others, 1930–31; Ely, 1933).

As regards the interpretation to be placed on the finding that there is an increased alkalinity of the blood in cancer, Reding and his co-workers conclude that the increased alkalinity is specifically related to the malignant disease. They found that the alkalinity is greater the more malignant the tumour, and that after surgical removal of the tumour, the blood reaction does not always return to normal. Furthermore, they report that many members of the families of subjects with cancer have a reaction of the blood that is more alkaline than normal. From these findings they conclude that the increased alkalinity is the cause, rather than the result, of the malignant growth, and that the reaction of the blood is of considerable value for prognosis. Woodward and her co-workers (1930–31) are in general agreement with these conclusions.

In contrast with these findings, it should be pointed out that the observations of many other workers give no support to the suggestion that cancer is associated with an increased alkalinity of the blood. Myers and Booher (1924) have investigated the acid-base equilibrium of a large number of hospital patients, and, although they find that an increased alkalinity is associated with certain pathological conditions, among which are mentioned fever and persistent vomiting, yet they do not find that an increased alkalinity is specifically related to malignant disease.

It is possible that the increased alkalinity found by some workers in cancer is due, not to the malignant disease as such, but to conditions consequent on the growth, such as cachexia, or disturbances of the alimentary tract. An increased alkalinity may be associated with a poor prognosis only because cachexia increases as the disease progresses. The absence of changes in blood reaction in the early stages of "surface cancer" is in accordance with this view. That a high blood pH may occur in pathological conditions other than cancer has been shown by Ely (1933) and by Myers and Booher (1924), and that cancer can occur without any increase of blood alkalinity has been shown by a number of workers (Myers and Booher, 1924; Corran and Lewis, 1924; Millet, 1929; Beregoff, 1930; Guthmann and Wirz, 1930; Mercier and Rossier, 1930; Woodard, 1930).

Reding and Slosse (1930, *a*) have put forward the hypothesis that the curative action of radiation is due, not to the direct action on the tumour, but to a general action on the body fluids. They say that radiation causes the reaction of the blood to return to a normal level within a few hours, and in this way brings about the cure of the malignant growth. In those cases in which the return to normal does not occur, the treatment is unlikely to be successful. McDonald (1927) reports similar findings. These results, however, are at variance with those of other workers, who have found that radiation results in an increased alkalinity of the blood (Myers and Booher, 1924; Warren, 1928).

The discrepancies between the results of various workers may, in part, be due to differences in the technique employed for the determination of the blood reaction. Most workers have used some form of colorimetric method. This has the disadvantage that it involves dilution of the blood.

The correction required in these circumstances is not constant, and is particularly liable to variation in pathological conditions (Bennett, 1926; Marrack and Thacker, 1926; Myers and Muntwyler, 1928; Johnston, 1928). Moreover, many of the investigators have made the measurements at room temperature, and applied a correction to obtain the pH at body temperature. This is subject to error, because the temperature coefficient of the pH of blood is not known accurately, and may be variable, particularly in pathological conditions. Values for the coefficient, varying from 0·01 pH to 0·015 pH per degree Centigrade, have been obtained (Martin and Lepper, 1926; Marrack and Thacker, 1926; Havard and Kerridge, 1929).

Measurements made by the glass electrode at body temperature are free from these sources of error. One worker (Millet, 1929) has used the glass electrode for his actual determinations, but he worked at room temperature. Errara, Reding and Slosse (1930) report general agreement between the glass electrode and their colorimetric method. Woodward and others (1930–31) have also checked their colorimetric method against the glass electrode.

Another consideration of importance in connection with technique is the method of obtaining blood for the determinations. Most previous workers have drawn blood from an arm vein. Now the reaction of venous blood from a limb is affected by: (*a*) the reaction of the arterial blood reaching the limb; (*b*) the rate of metabolism of the limb; and (*c*) the rate of circulation through the limb. Both (*b*) and (*c*) are variable, and are affected by the temperature of the limb and its surroundings. The reaction of the blood in an arm vein, therefore, does not necessarily give an accurate indication of the reaction of the blood and tissues of the body generally.

These considerations led us to make a short series of determinations of the reaction of the arterial blood of hospital patients, with and without malignant disease, the determinations being made by means of the glass electrode and at body temperature.

TECHNIQUE.

Eight to 10 c.c. of blood were withdrawn from the radial artery of each subject into a syringe containing 1 c.c. of a solution of 3 per cent. potassium oxalate and 2 per cent. sodium fluoride (neutralized). This solution prevented clotting and glycolysis, and also ensured that the blood did not come into contact with air in the dead space of the syringe. The small amount of dilution by the neutralized solution (1 part of solution to 9 of blood) has no appreciable effect on the reaction of the blood. The junction between the barrel and the plunger of the syringe and the junction between syringe and needle were both sealed with liquid paraffin to prevent air from leaking into the syringe. The small bubble of air in the needle prevented the oxalate and fluoride solution from coming into contact with the tissues of the subject, and was too small to affect the reaction of the sample of blood. The blood was transferred from the ward to the laboratory in the syringe at room temperature. If there were any delay in measuring the pH, the syringe was put in the ice-chest.

The determinations were made within one or two hours from the time of withdrawal of the blood, except in the case of one duplicate measurement.

Since the pH was not measured immediately after withdrawal of the blood from the artery, the possibility of a change of reaction after the blood is shed is a matter of some importance. A change due to glycolysis would be inhibited by the fluoride, but there remains to be considered the " acid change " described by Havard and Kerridge (1929). Now, in earlier experiments with the types of apparatus used for the present work, in which freshly shed blood was immediately transferred to the electrode vessel, no acid change was observed of the type described by Havard and Kerridge. These observations have been reported in a previous paper (Dickinson, Havard and Platt, 1933), and it was pointed out that an apparent change, such as Havard and Kerridge described, could result from a warming up of the blood after it was transferred to the electrode vessel.* Additional control experiments with blood obtained by the technique described above have shown that there is no appreciable alteration in reaction when the blood is kept at room temperature for an hour or in the ice-chest for twenty-four hours.

Two different forms of glass electrode were employed for the determination of the pH of the blood. One form was that introduced by Stadie, O'Brien and Laug (1931), and it was used in conjunction with a Lindemann electrometer (Kerridge, 1926). With this apparatus it was possible to read to 0·2 m.v. (corresponding to 0·003 pH), and the measurement could be made on 1 c.c. of blood. The second instrument was that described by Platt and Dickinson (1933), and a valve electrometer was used to make the potential measurements. With this it was possible to read to 0·1 m.v. (corresponding to 0·0015 pH), but 4 c.c. of blood were required for an accurate determination. Temperature regulation was obtained in the first apparatus by means of a water-bath, and in the second by means of an air-bath, each kept at 37° C. Before the blood was introduced into the apparatus in the air-bath, the syringe containing the blood was warmed in a beaker of water at 37° C. With the Stadie form of electrode this was unnecessary, since temperature equalization was rapid, the potential readings indicating that equilibrium was reached within five minutes. Errors due to inequalities of temperature were reduced to a minimum, since N/10 HCl was used as the standard solution on the side of the membrane opposite to the blood (Dickinson, Havard and Platt, 1933). In both instruments N/10 HCl quinhydrone electrodes were used as half-cells.

Before making a determination of the pH of blood, the apparatus was standardized by taking readings with a phosphate buffer of known pH in the electrode vessel. When possible, duplicate measurements were made on separate portions of the same sample of blood. Of ten duplicate measurements, in which one determination was made with one apparatus and one with the other, six agreed to within 0·01 pH, three differed by more than 0·01 pH, but agreed to within 0·02 pH, and one gave a difference of 0·022 pH.

* Platt and Dickinson (1933), in work on the technique of glass electrode measurements, have discussed the difficulties of accurate temperature control, and have shown that, if a fluid is introduced into the electrode vessel at a temperature above or below that of the glass-cell system, an apparent alkaline or acid change will be observed. The direction and magnitude of such a change will depend on the composition of the fluid introduced and on that of the standard solution on the other side of the glass membrane.

RESULTS.

The results are shown in Table I. The first column gives the pH of the arterial blood of patients with either epithelioma or secondary glands arising from epitheliomata; the second column gives that of patients of the cancer-bearing age not suffering from cancer, and the third column gives that of five younger subjects—three laboratory workers and two hospital patients. The pH values have been arranged in numerical order, with the more acid above and the more alkaline below. The condition of the subjects without cancer is indicated in the table. There is no *à priori* reason for supposing that the complaints from which they suffered should affect the acid-base equilibrium.

TABLE I.—*The pH of the Arterial Blood of Subjects with Cancer and of Control Subjects.*

Subjects with cancer.			Controls.					
			Age over 40.			Age under 40.		
Age.	pH.	Description.	Age.	pH.	Description.	Age.	pH.	Description.
57	7·395	Secondary glands.	48	7·420	Fracture.	25	7·405	Lab. worker.
56	7·415	Carcinoma of tongue.	50	7·420	Foot manipulated.	27	7·410	,, ,,
			51	7·420	Fracture.	32	7·415	,, ,,
56	7·420	Secondary glands.	57	7·425	Cataract.	35	7·430	Cellulitis of leg.
54	7·430	,, ,,	68	7·435	Fracture.			
65	7·440	Carcinoma of penis.	57	7·445	Cataract.	35	7·440	Varicose veins.
			64	7·470	Hydrocele of cord.			
57	7·445	Epithelioma of ear.	65	7·470	Osteo-arthritis.			
60	7·445	,, of finger.						
84	7·445	Carcinoma of palate.						
65	7·465	Secondary glands.						
	7·430	Mean of 9.		7·440	Mean of 8.		7·420	Mean of 5.

It is seen from Table I that there is no significant difference between the pH of the arterial blood of patients with surface cancer or secondary glands, and that of patients of corresponding ages with other complaints. Table I also shows a fairly wide range of variation in the measurements from subjects of the first and second groups. The reaction of the arterial blood depends finally on the activity of the respiratory centre; a momentary interference with the normal functioning of the centre may possibly give rise to alterations in the reaction of the blood.

An experiment was therefore made to determine the order of magnitude of the alterations in the pH of arterial blood that could be brought about by holding the breath or by over-breathing. The results are shown in Table II. The samples of blood taken while the subject was resting and while holding the breath were obtained from the radial artery. When the subject was over-breathing the radial artery was so sensitive that it closed down on the needle and blood could not be obtained; the blood was therefore taken from an arm vein with the arm immersed in water at 47° C. (Meakins and Davies, 1920).

TABLE II.—*The Effect of Alterations of Respiration on the pH of the Blood.*

Condition of subject.	pH of blood.	Alveolar CO_2 percentage.
Resting	7·415 (arterial blood)	5·75
Holding breath	7·370 ,, ,,	7·10
Overbreathing (2 minutes)	7·570 ("arterialized venous blood")	2·45

The results show that the pH of arterial blood, or of "arterialized venous blood", is considerably affected by disturbances of respiration. Thus, it is possible to obtain large momentary variations in the reaction of arterial blood. In view of this, it is not possible to place much reliance on the pathological significance of small differences in blood reaction.

SUMMARY.

1. The results of previous workers are summarized and discussed.

2. The technique is described of collection of samples of arterial blood from patients with and without malignant diseases, and the measurement of the pH of the blood at 37° C, by two independent glass electrodes.

3. The results support the conclusion that the blood of patients with malignant disease of the mouth or skin, or with secondary glands, is not more alkaline than that of patients free from malignant disease.

4. An experiment is described that demonstrated that momentary alterations of respiration can affect the pH of the blood.

We wish to express our gratitude to Mr. A. A. Digges la Touche, Surgeon in charge of the Radium Clinic of the General Infirmary at Leeds, for his interest and help in the selection of subjects for this work.

REFERENCES.

BENNETT, M. A.—(1926) *J. Biol. Chem.*, **69**, 693.
BEREGOFF, P.—(1930) *J. Cancer Res.*, **14**, 559.
BISCHOFF, F., LONG, M. L., AND HILL, E.—(1930) *J. Pharmac.*, **39**, 425.
CHAMBERS, W. H.—(1923) *J. Biol. Chem.*, **55**, 229.
CORRAN, J. W., AND LEWIS, W. C. M.—(1924) *Biochem. J.*, **18**, 1358.
DICKINSON, S., HAVARD, R. E., AND PLATT, B. S.—(1933) *J. Physiol.*, **78**, 28P.
ELY, J. O.—(1933) *Amer. J. Cancer*, **17**, 58.
ERRARA, J., REDING, R., AND SLOSSE, A.—(1930) *C. R. Soc. Biol.*, **103**, 24.
GUTHMANN, H., AND WIRZ, P.—(1930) *Arch. Gynäk.*, **142**, 530.
HAVARD, R. E., AND KERRIDGE, P. M. T.—(1929) *Biochem. J.*, **23**, 600.
HUEPER, W. C., WOODWARD, G. E., AND FRY, E. G.—(1931) *Amer. J. Cancer*, **15**, 2666.
JOHNSTON, C. G.—(1928) *J. Biol. Chem.*, **79**, 297.
KERRIDGE, P. M. T.—(1926) *J. Sci. Instr.*, **3**, 404.
MCDONALD, E.—(1927) *Med. J. Rec.*, N.Y., **125**, 795.
MCDONALD, E., AND HUEPER, W. C.—(1930–31) *J. Lab. Clin. Med.*, **16**, 713.
MARRACK, J., AND THACKER, G.—(1926) *Brit. J. Exp. Path.*, **7**, 265.
MARTIN, C. J., AND LEPPER, E. H.—(1926) *Biochem. J.*, **20**, 1071.
MEAKINS, J., AND DAVIES, H. W.—(1920) *J. Path. Bact.*, **23**, 451.

MERCIER, P., AND ROSSIER, P. H.—(1930) *Le Cancer*, **7**, 249.
MILLET, H.—(1929) *J. Biol. Chem.*, **82**, 263.
MYERS, V. E., AND BOOHER, L. E.—(1924) *Ibid.*, **59**, 699.
MYERS, V. E., AND MUNTWYLER, E.—(1928) *Ibid.*, **78**, 243.
PLATT, B. S., AND DICKINSON, S.—(1933) *Biochem. J.*, **27**, 1069.
REDING, R.—(1928) *Cancer*, Bruxelles, **5**, 97.
Idem, AND SLOSSE, A.—(1930, *a*) *C.R. Soc. Biol.*, **103**, 27.—(1930, *b*) *Ibid.*, **104**, 124.
STADIE, W. C., O'BRIEN, H., AND LAUG, E. P.—(1931) *J. Biol. Chem.*, **91**, 243.
WARREN, S. L.—(1928) *Physiol. Rev.*, **8**, 92.
WOODARD, H. Q.—(1930) *J. Cancer Res.*, **14**, 319.
WOODWARD, G. E., SCHOONOVER, J. W., FRY, E. G., TORRANCE, E. G., AND MCDONALD, E.—(1930–31) *J. Lab. Clin. Med.*, **16**, 704.

THE DIFFERENTIATION OF THE VIRUS OF VESICULAR STOMATITIS FROM THE VIRUS OF FOOT-AND-MOUTH DISEASE BY FILTRATION.*

I. A. GALLOWAY AND W. J. ELFORD.

From the National Institute for Medical Research, London.

Received for publication October 7th, 1933.

VESICULAR stomatitis is a disease affecting both horses and cattle under natural conditions. It possesses some superficial resemblance to foot-and-mouth disease in that, with the possible exception of the horse, the same animals are susceptible to both, while the lesions produced by the two diseases are indistinguishable. Almost all the experimental work on vesicular stomatitis has been done in America, where notably Cotton (1926, *a*; 1926, *b*; 1927), Olitksy, Traum and Schoening (1926), and Olitsky (1927), have shown the disease to be due to a filterable virus, and have accumulated much evidence upon animal susceptibility and the development of immunity to the disease. These authors showed that the virus would pass readily through the more porous grades of filter-candles, Berkefeld V and N, and Chamberland L3, and also a Seitz filter. Berkefeld W and Chamberland L7 candles retained all but a very small proportion of the virus. Olitsky (1927) stated that the filterability of the virus paralleled that of foot-and-mouth disease ; " the indications are that it conforms with that of foot-and-mouth disease in respect of magnitude, charge and isoelectric range."

Full accounts of the experimental disease, and the several grounds which have been suggested for differentiating vesicular stomatitis from foot-and-mouth disease, are contained in the references cited at the end of this paper. The chief contrasts in the characteristics of these two diseases have been set out somewhat categorically in Table I. It is perhaps advisable to discuss in

* This work was done on behalf of the Foot-and-Mouth Disease Research Committee, who have kindly given their permission for the publication of this paper.

more detail the question of the contagiousness of the diseases, and also cross-immunity tests. The earlier observations of Mohler (1918) and others pointed to the fact that vesicular stomatitis is less contagious than foot-and-mouth disease, but reports of more recent outbreaks of the former disease among cattle in America suggest that this characteristic is not always maintained. Cotton (1927), referring to an outbreak in New Jersey, noted that 33 different herds were affected in an area of 300 sq. miles and 700 animals developed the disease.

As regards cross-immunity tests, Olitsky, Traum and Schoening (1926) showed that cattle and guinea-pigs recovered from type Vallée A or type Vallée O foot-and-mouth disease virus were still susceptible to the strain of vesicular stomatitis with which they worked. Similar cross-immunity tests with the type C foot-and-mouth disease virus and the virus of vesicular stomatitis have not been made. Since the fact has been established that there are at least three types of the virus of foot-and-mouth disease and two of the virus of vesicular stomatitis, " New Jersey " and " Indiana " (Cotton, 1927), such cross-immunity tests have become very complicated. The question is not simplified when it is realized that there appear to be some strains believed to be foot-and-mouth disease which cannot be definitely typed, and there may exist other unrecognized types of the virus of vesicular stomatitis.

The differentiating features of the two diseases, set out in Table I, are not absolute, or such as would wholly exclude the possibility of the virus of vesicular stomatitis being a variant of the virus of foot-and-mouth disease, with which it has up to the present been considered to have some generic relationship.

Recent experiments, conducted in collaborations with Dr. F. M. Burnet, have demonstrated that the " Indiana " strain of the virus of vesicular stomatitis will survive and propagate in the chorio-allantoic membrane of the egg. The virus of foot-and-mouth disease, on the other hand, has been found not to survive under similar conditions. Full details of these experiments and the technique employed, a modification of that described by Woodruff and Goodpasture (1931), will be given later.

The object of the present investigation has been to determine the size of the elementary virus particles of vesicular stomatitis, for comparison with the previously determined value for the virus of foot-and-mouth disease. In this way it was hoped to establish a more definite basis for differentiating the two diseases.

EXPERIMENTAL.

The size of the virus of foot-and-mouth disease has already been estimated by ultrafiltration analysis to be 8–12mμ. (Galloway and Elford, 1931), and the same technique of filtration through gradocol membranes (Elford, 1931) has been adopted in the present study.

Strains of Virus.

Two strains of the virus of vesicular stomatitis have been studied, the " Indiana " strain and the " New Jersey " strain. These were kindly supplied to us by Dr. W. E. Cotton, of the U.S.A. Bureau of Animal Industry. Some

TABLE I.—*Contrast in Characteristics of Vesicular Stomatitis and Foot-and-Mouth Disease.*

Contrast.	Vesicular stomatitis.	Foot-and-mouth disease.
Susceptible animals	Horses susceptible.	Horses very, if not absolutely resistant to infection.
Active immunity	Subinfective doses of virus produce a solid immunity.	Subinfective doses of virus do not, as a rule, produce an immunity.
Passive immunity	Immune serum will protect against the primary lesion in the guinea-pig.	Immune serum will not protect against the primary lesion in the guinea-pig.
Contact infection: Cattle	Contact infection not easy to produce experimentally.	Very contagious.
Contact infection: Pigs	Infection of *young* pigs by contact possible.	,, ,,
Contact infection: Guinea-pigs	Guinea-pigs may develop an immunity through contact, although developing no visible lesions.	Guinea-pigs do not develop an immunity by contact unless lesions appear.
Generalized lesions	Occur infrequently. In the guinea-pig the generalized lesions tend to appear on the uninoculated fore pads and the tongue is only rarely involved.	Occur with great regularity. (Some strains are reported to behave irregularly in this respect.) Most strains give generalized lesions on the tongue as well as on the uninoculated pads of guinea-pigs.
Appearance of lesions in guinea-pigs	Fully developed primary lesions on pads 48–72 hours after inoculation.	Fully developed primary lesions 24 hours after inoculation.
Neurotropism	Symptoms referable to lesions in central nervous system follow intra-cerebral inoculation of virus into guinea-pigs and mice.	No evidence of symptoms or lesions referable to the central nervous system.
Tissue culture	Evidence of survival and propagation in tissue cultures of chick embryo pulp.	No evidence of survival of virus in tissue cultures of chick embryo pulp.

notes on the survival of the virus may be made here, since previous attempts to obtain active material from America were unsuccessful. On these occasions the material, consisting of pads removed from infected guinea-pigs, was sent in (*a*) 50 per cent. glycerine and physiological saline ; (*b*) 50 per cent. glycerine and phosphate buffer at pH 7·6 ; (*c*) without any addition of liquid ; and (*d*) in the form of a powder prepared from infected pads which had been dried *in vacuo*. The time of transit by post would be about 10 days, but on arrival all such samples proved to be non-infective for guinea-pigs. The failure of vesicular stomatitis to survive under these conditions is a point worthy of note, since it is in contrast to the general experience with the virus of foot-and-mouth disease which has usually been found to survive when sent in a 50 per cent. glycerine medium.

Sir Henry Dale kindly brought the " Indiana " strain when returning from America. The material was placed in cold storage on the liner during the journey. The " New Jersey " strain came by post. In both instances the pads from infected guinea-pigs were sent (*a*) in 50 per cent. glycerine and physiological saline, (*b*) in 50 per cent. glycerine and acid potassium phosphate at pH 7·6, and (*c*) with only a very small quantity of physiological saline added. The material found to be infective for guinea-pigs was sample (*c*) with both strains, although this was putrefying. Both strains have since been maintained in guinea-pigs.

Lesions in Guinea-pigs.

Guinea-pigs inoculated intradermally in the pads with a sufficient dose of virus may show small lesions along the track of the needle as early as the 24th hour, but in many instances they do not appear until from the 36th to the 48th hour. The lesions are not fully developed until the 48th to the 60th hour. Our experience has been that the amount of fluid contained in the fully developed vesicles is generally considerably less than in the case of infection with the various strains of foot-and-mouth disease. Some evidence has also been obtained corroborative of that reported by Long and Olitsky (1928) and Wagener (1932, *a*), that guinea-pigs which have received a subinfective dose of virus frequently prove to be actively immune—a phenomenon which is of rare occurrence in the case of foot-and-mouth disease in guinea-pigs. Generalized lesions have appeared in less than 50 per cent. of inoculated guinea-pigs. In only two cases have these appeared on the tongue. When present they have appeared on one or both of the fore pads.

Source of Virus and Preparation of Stock Virus Suspensions.

The lesions appearing at the site of the intradermal inoculation in the pads of guinea-pigs provide a vesicular fluid of high virus content. This fluid supplemented by the skin removed from the lesions has served as the source of virus. The vesicular fluid and skin from the lesions has been ground up with powdered glass in Hartley's broth at pH 7·6 to furnish an emulsion containing approximately 5 per cent. of infective tissue material. This crude emulsion was centrifugalized for 5 minutes at 2500 revs. per min., and the supernatant liquid pipetted off and filtered through a standardized sand and

pulp filter. In this way the emulsion was cleared of coarsely suspended material, and was then filtered through a permeable membrane of A.P.D.* 0·4μ to 0·6μ, using a positive pressure of 20 cm.Hg. to furnish a stock filtrate of high virus potency free from ordinary bacteria. This has been the routine procedure for obtaining a virus suspension suitable for further examination by ultra-filtration analysis.

Method of Testing Filtrates.

Filtrates were tested for presence of virus by making intradermal inoculations into the pads of two guinea-pigs. In certain crucial tests, *e. g.* after filtration through membranes of A.P.D. 0·13μ, four guinea-pigs were used. In those instances where the test produced no reaction in the inoculated guinea-pigs, the animals were subsequently tested for immunity such as may follow a subinfective dose of virus. In no case was a guinea-pig which had failed to react to the inoculation of undiluted filtrate found to possess any demonstrable immunity. It is therefore reasonable to conclude that the filtrates producing no reactions were completely free of virus. In estimating the amount of virus in a filtrate, *i. e.* the degree of infectivity, tests were made with serial tenfold dilutions of the liquid. The concentration of virus is expressed in terms of the limiting dilution producing lesions. This is referred to as the titre of the filtrate.

Potency of Stock Filtrate.

Stock filtrates prepared from broth emulsions of 48-hour vesicle lymph and pad tissue, and using membranes of A.P.D. 0·4μ–0·6μ, have possessed consistently titres between 1 : 10,000 and 1 : 100,000. When the lymph used was collected after 24 hours the titre was somewhat lower, viz. 1 : 1000 to 1 : 10,000. It may be noted here that filtrates of the virus of foot-and-mouth disease prepared in a comparable manner invariably prove infective in a dilution of 1 : 100,000, and sometimes 1 : 1,000,000.

In addition a stock filtrate of both strains of the virus of vesicular stomatitis was prepared using a Seitz filter instead of a collodion membrane, all other conditions being the same as above. The volume of filtrate collected was in each case 20 c.c., and the titres of both filtrates were 1 : 10,000.

Cross-immunity Tests with the Two Strains of Virus Used.

In order to make certain that the two strains of virus actually used in these experiments were immunologically distinct, some cross-immunity tests were made. Guinea-pigs were infected separately with the "Indiana" and "New Jersey" strains respectively, and when the lesions had healed were re-inoculated with the homologous strain of virus. The animals proved to be immune to the homologous strain. Upon subsequent inoculation with the heterologous strain the guinea-pigs developed typical vesicular lesions, thus showing their susceptibility to the heterologous strain.

Guinea-pigs immune to either of the two strains of vesicular stomatitis

* A.P.D. = Average pore diameter.

were still susceptible to the G.F. strain (Vallée O type) of the virus of foot-and-mouth disease, while guinea-pigs immune to this strain of foot-and-mouth disease were susceptible to both strains of vesicular stomatitis.

The End-point in Membrane Permeability and Estimated Size of the Virus.

The early experiments were designed to provide an answer to the question as to whether or not the end-point in membrane permeability for the virus of vesicular stomatitis was the same as that for the virus of foot-and-mouth disease. Knowing the end-point of membrane permeability for the latter virus to be A.P.D. 25mμ. (Galloway and Elford, 1931), a stock filtrate of the virus of vesicular stomatitis, "Indiana" strain, was filtered through membranes of grades 24 mμ., 35 mμ. and 45mμ. respectively. In each case the virus was completely retained by these membranes, whereas the virus of foot-and-mouth disease passes membranes of grade 35mμ. and 45mμ. readily. In the next experiment, phages S13 and C36, the end-points for which are A.P.D. 25 mμ. and 60 mμ. respectively (Elford and Andrewes, 1932), were added to a stock filtrate of the virus of vesicular stomatitis, and the mixture was filtered at a pressure of 76 cm.Hg. through membranes of grades 99mμ., 60 mμ. and 45mμ. Tests upon the filtrates furnished the results given in Table II.

TABLE II.—*Filtration of Mixture of Virus of Vesicular Stomatitis and Phages S13 and C36.*

Membrane A.P.D. in mμ.	Tests on filtrate. S13.	C36.	Virus.	Remarks.
45	+	—	—	*Mixture:* Virus titre = 1 : 10,000. S13 $2{\cdot}5 \times 10^6$/c.c. C36 $2{\cdot}5 \times 10^7$/c.c.
	1×10^5/c.c.			
60	+	—	—	
	4×10^5/c.c.			
99	+	+	—	
	1×10^6/c.c.	5×10^5/c.c.		

The phage was estimated by the plaque count method on agar, and the concentration is expressed as the number of particles per c.c.

These experiments at once sufficed to demonstrate that the virus of vesicular stomatitis, "Indiana" strain, differs in size very considerably from the virus of foot-and-mouth disease, and is even larger than C36 phage, which has a diameter of 20–30mμ.

The exact end-point for the virus was next determined, and the experimental results obtained by filtering stock filtrates of the "Indiana" strain of the virus through membranes of various grades are given in Table III. It will be seen that membranes of an A.P.D. of 0·35μ yielded filtrates containing the virus in undiminished concentration as compared with the original, but a considerable drop in infectivity was shown by filtrates from membranes of A.P.D. of 0·2μ, and a still greater diminution characterized the filtrates from

TABLE III.—*Filtration of the Virus of Vesicular Stomatitis through Membranes of Graded Porosities.*

Strain of virus.	Limiting infective dilution of stock filtrate	Membrane A.P.D. in mμ.	Volume filtered per sq. cm.	Number of experiments.	Test on filtrate for presence of virus and titre.	Remarks.
"Indiana"	1 : 10,000 to 1 : 100,000	350	10	2	+ 1 : 10,000	Fluid filtered was in each case a stock filtrate from a membrane of A.P.D. 0·4μ to 0·6 μ. Filtration pressure = 76 cm. Hg. Membrane thickness = 0·1–0·15 mm. Reference should be made to Table I for results with membranes of grades less than A.P.D. 130 mμ.
		200	10	2	+ 1 : 100 to 1 : 1000	
		160	10	4	+ 1 : 10 to 1 : 100	
	1 : 1000*	160	10	1	Negative	
	1 : 10,000 to 1 : 100,000	130	10	3	,,	
"New Jersey"	1 : 10,000 to 1 : 100,000	160	10	2	+, only tested undiluted	
	1 : 1000*	160	10	1	Negative	
	1 : 10,000 to 1 : 100,000	130	10	4	,,	
	1 : 1000*	130	10	1	,,	

* These stock filtrates were prepared from material collected from lesions at the 24th hour. The low initial concentration of virus in these filtrates would account for the negative results in tests on filtrates from membranes of an A.P.D. of 160 mμ.

membranes of an A.P.D. of 0·16μ. The filtrates from membranes of an A.P.D. of 0·13μ or less were consistently non-infective.

Parallel experiments were made with the " New Jersey " strain of virus which was received from America when the experiments with the " Indiana " strain were nearing completion. As will be seen from the results given in Table III, the filtrates from membranes of an A.P.D. of 0·13μ were non-infective while the virus passed through the membranes of an A.P.D. of 0·16μ.

These results, interpreted on the basis of the method outlined in a previous paper (Elford, 1933), indicate the particle size of the virus to be 70–100mμ. This figure is about eight times that determined for the virus of foot-and-mouth disease (8–12 mμ.). Such a vast difference in the physical properties of the viruses points directly to the distinct individuality of the two diseases, and would be extremely difficult to reconcile with a " variant " hypothesis.

Effect of Hydrogen Ion Concentration on Filtration.

Olitsky (1927) stated that the virus would pass regularly through Chamberland L11 filter candles when suspended in a buffered phosphate-saline medium of which the hydrogen ion concentration was on the alkaline side of pH 8, but that it would not do so when the hydrogen-ion concentration was pH 7·5. One experiment has been made with graded collodion membranes to study the effect on filtration of varying the pH of the broth medium in which the virus is suspended. The technique employed was as follows :—Three batches of Hartley's broth were made up at pH 6·5, pH 7·6 and pH 8·4. In varying the pH value of the broth medium N/10 hydrochloric acid and N/10 sodium hydroxide were used. A stock virus filtrate was made up in the usual way. A quantum of this virus filtrate was added to each of the three samples of broth in the proportion of 1 of virus filtrate to 4 of broth, so that each system contained initially the same amount of virus. The actual pH values of the system were checked electrometrically. Ten c.c. amounts of each system were filtered through membranes of an A.P.D. of 0·13μ, the end-point for the virus. Each of the three filtrates was inoculated intradermally into the pads of four guinea-pigs. None of these developed lesions, nor did they subsequently prove immune to an intradermal pad inoculation of a strong dose of virus.

Titration of each of the stock systems, the respective pH values of which were finally at pH 6·8, 7·6 and 8·25, was also made by inoculation of ten-fold dilutions intradermally into the pads of guinea-pigs. The titre of each proved to be about 1 : 10,000. There was thus no evidence that the potencies of the virus suspensions at pH 6·8 to 8·25 fell off appreciably during the period necessary for the experiment—approximately 4 hours— and the variation of hydrogen ion concentration over the range 6·8 to 8·25 had no effect on the filtration of the virus in a broth medium.

SUMMARY.

It has been shown that the virus of vesicular stomatitis has a particle size of 70–100mμ. This size value, which is the same for both the " Indiana " and " New Jersey " strains of the virus, is about eight times that previously

estimated for the virus of foot-and-mouth disease (8–12 mμ.). This is regarded as direct evidence of the distinct individuality of the two diseases.

REFERENCES.

CARREL, A., OLITSKY, P. K., AND LONG, P. H.—(1928) *C. R. Soc. Biol.*, **98**, 827.

COTTON, W. E.—(1926, *a*) *J. Amer. Vet. Ass.*, **69**, 313.—(1926, *b*) *Ibid.*, **70**, 168.—(1927) *Vet. Med.*, **22**, 169.

COX, H. R., AND OLITSKY, P. K.—(1933) *Proc. Soc. Exp. Biol. Med.*, **30**, 653.—(1933) *Ibid.*, **30**, 654.

COX, H. R., SYVERTON, J. J., AND OLITSKY, P. K.—(1933) *Ibid.*, **30**, 896.

ELFORD, W. J.—(1931) *J. Path. Bact.*, **34**, 505.—(1933) *Proc. Roy. Soc.*, B, **112**, 384.

GALLOWAY, I. A., AND ELFORD, W. J.—(1931) *Brit. J. Exp. Path.*, **12**, 407.

HOFFMANN, D. C.—(1931) *J. Exp. Med.*, **53**, 43.

LONG, P. H., AND OLITSKY, P. K.—(1928) *Proc. Soc. Exp. Biol. Med.*, **25**, 478.

MAITLAND AND MAITLAND.—(1931) '4th Progress Report, Foot and Mouth Disease Research Committee,' London (H.M. Stationery Office).

MOHLER, J. R.—(1918) *J. Amer. Vet. Ass.*, **52**, 410.

OLITSKY, P. K.—(1927) *J. Exp. Med.*, **45**, 969.

Idem AND LONG, P. H.—(1928) *Proc. Soc. Exp. Biol. Med.*, **25**, 287.—(1928) *J. Exp. Med.*, **47**, 835.

OLITSKY, P. K., TRAUM, J., AND SCHOENING, H. W.—(1926) *J. Amer. Vet. Med. Ass.*, **70**, 147.

OLITSKY, P. K., COX, H. R., AND SYVERTON, J. T.—(1933) *Science*, **77**, 611.

WAGENER, K.—(1931 *a*), *Vet. Med.*, **26**, 388.—(1931, *b*) *Cornell Vet.*, **21**, 344.—(1932, *a*) *J. Amer. Vet. Med. Ass.*, **80**, 39.—(1932, *b*) *Ibid.*, **80**, 579.—(1932, *c*) *Ibid.*, **81**, 160.

THE EFFECT OF REPEATED INJECTIONS OF A BACTERIAL VACCINE.

W. W. C. TOPLEY.

From the London School of Hygiene and Tropical Medicine.

Received for publication November 3rd, 1933.

IN previous papers (Greenwood, Topley and Wilson, 1931, *a*, *b*) we have described attempts to immunize mice against *Bact. aertrycke*. Using a vaccine prepared from a smooth virulent strain, and giving two injections of 500×10^6 bacilli at a week's interval, it was found, in conformity with the results recorded by several other workers, that a partial immunity could be induced. The immunized mice were significantly more resistant to subsequent experimental infection with a single dose of living bacilli than normal, non-immunized controls. Such immunized mice were also more resistant than their normal companions to contact infection in an infected herd, in that they lived, on the average, significantly longer. The immunity conferred was, however, far from complete. Most of the immunized mice died eventually from *Bact. aertrycke*

infection, and the immunization of all entrants to an infected herd for a period of one year failed entirely to bring to a close the epidemic prevalence from which the herd was suffering. These results were in accord with our earlier experience (Topley, 1921 ; Topley, Ayrton and Lewis, 1924 ; Topley, Wilson and Lewis, 1925 ; Greenwood and Topley, 1925 ; Greenwood, Newbold, Topley and Wilson, 1926, 1928, 1930), which indicated that the natural immunity acquired by exposure to epidemics of mouse typhoid is seldom, if ever, of a kind that enables mice to withstand indefinitely the repeated contact infections to which they are exposed under these conditions.

In view of these results it seemed of interest to determine the effect of repeated inoculations with a killed vaccine similar to that employed in the earlier experiments.

EXPERIMENTAL PROCEDURE.

The vaccine employed was prepared from a smooth virulent strain of *Bact. aertrycke*. It was killed by adding 0·25 per cent. formalin to a thick bacterial suspension in saline, followed by heating for 1 hour at 55° C. The same preparation was used throughout. It was stored in the ice-chest, and diluted for use as required. Each injection consisted of 500×10^6 bacilli contained in 0·5 c.c. of saline. All injections were given intraperitoneally at weekly intervals. A large number of mice (450) received the first injection. Each week thereafter, up to the fourth week, 30 mice were withdrawn from the immunized group and tested for resistance, together with 30 non-immunized controls. The remaining mice were given another injection of vaccine. These weekly immunizing injections were continued until the thirteenth week, further samples being withdrawn for testing after 6, 8, 12 and 13 injections, The test dose of living virulent *Bact. aertrycke* was in all cases 1000 bacilli, contained in 0·25 c.c. of Ringer's solution and injected intraperitoneally. All mice, after inoculation, were housed in separate cages, and were observed for 28 days. All mice that died were submitted to *post-mortem* examination, including cultures from the spleen and heart's blood. In almost every case *Bact. aertrycke* was isolated. All mice alive on the twenty-eighth day were killed and submitted to the same *post-mortem* examination as those that died.

The results are summarized in Table I.

DISCUSSION.

Three criteria were available in these experiments for assessing the grade of resistance of the immunized mice.

It has been a common experience in earlier experiments that mice with a low grade of immunity tend to develop, after experimental or contact infection, a subacute but fatal illness, associated with gross enlargement of the spleen and multiple lesions in the liver, in place of the more acutely fatal bacteræmic infection that is usual in non-immunized mice. This effect may be measured by comparing the mean time to death of those mice that die in the immunized and control groups. This comparison is made in the first three columns of Table I, the third column giving the differences and their standard errors.

A higher grade of immunity is characterized by the survival of a considerable proportion of the immunized mice in apparent health, although a number

TABLE I.

Number of times vaccinated.	Mean time to death (days).			Per cent. survivors.			Per cent. completely resistant.		
	Vaccinated.	Controls.	Difference.	Vaccinated.	Controls.	Difference.	Vaccinated.	Controls.	Difference.
1	15·65	5·07	10·58 ± 1·612	43·33	0·00	43·33 ± 10·630	16·67	0·00	16·67 ± 7·136
2	15·00	5·21	9·79 ± 1·472	70·00	3·33	66·67 ± 12·445	46·67	0·00	46·67 ± 10·920
3	10·57	7·32	3·25 ± 2·328	76·67	6·67	70·00 ± 12·728	26·67	0·00	26·67 ± 8·775
4	21·80	7·46	14·34 ± 2·047	83·33	13·33	70·00 ± 12·904	70·00	0·00	70·00 ± 12·328
6	19·40	6·55	12·85 ± 2·142	83·33	3·33	80·00 ± 12·795	56·67	0·00	56·67 ± 11·635
8	14·10	8·21	5·89 ± 1·875	66·67	6·67	60·00 ± 12·443	33·33	3·33	30·00 ± 9·991
12	19·07	6·41	12·66 ± 1·476	53·33	10·00	43·33 ± 12·012	20·00	0·00	20·00 ± 7·746
13A	15·56	5·00	10·56 ± 1·395	46·67	3·33	43·34 ± 11·180	13·33	3·33	10·00 ± 7·136
13B	15·21	5·58	9·63 ± 1·520	36·67	3·33	33·34 ± 10·329	10·00	0·00	10·00 ± 5·627
13 (*Past.*)	6·32	7·71	−1·39 ± 1·222	15·00	22·50	−7·50 ± 8·728	12·50	5·00	7·50 ± 6·319

Each vaccinated or control group consisted of 30 mice, except in test 13 (*Past.*), where there were 40 vaccinated and 40 controls.

of these survivors may be suffering from latent infections, as revealed by the isolation of *Bact. aertrycke* from the spleen when they are killed and examined *post mortem*. The different samples of immunized mice may be graded according to this test by noting the differences between the percentage of survivors on the twenty-eighth day in the immunized and control groups. The relevant figures are given in columns 4–6 of Table I.

The highest grade of resistance is evidenced by mice that survive for 28 days after the test inoculation and, when killed and examined *post-mortem*, reveal no evidence of latent infection. For our present purpose such mice may be labelled as completely resistant, without any implication that they would in fact withstand all risks of specific infection. The proportions of such completely resistant mice in the vaccinated and control groups, and the differences between them, are set out in columns 7–9 of Table I.

Taking the rows of the Table in order, it is clear that a single immunizing injection has produced some degree of increased resistance, evidenced by a lengthening of the time to death of those mice that die, a significantly increased survival rate, and the complete resistance of a few of the treated mice. Passing down the Table it will be noted that, up to a total of 4 to 6 immunizing injections, the degree of immunity shows a general tendency to increase with the number of injections received, as judged by the percentage of survivors on the twenty-eighth day, or by the proportion of completely resistant mice. The effect as regards the lengthening of the time to death of those mice that die varies little with repeated immunizing injections—a fact that is in no way surprising.

The effect of 4 or 6 immunizing injections is, it will be noted, very considerable. In each case 83·33 per cent. of the mice survive infection, and the percentage of completely resistant mice is high—70 per cent. and 56·67 per cent. respectively. It may be noted that these percentages are significantly higher than those recorded in many earlier experiments with twice-vaccinated mice, described elsewhere. The twice-vaccinated mice in this particular series of tests gave unusually favourable results.

The resistance of the mice that received 8 immunizing injections is, however, less than that of those that received 6, and the resistance of those that received 12 less than that of those that received 8. The 12-times-vaccinated mice are, indeed, little if at all more resistant than those that received a single injection. Since this result was unexpected, the next test, with mice that had received 13 immunizing injections, was performed in duplicate, using two different cultures of virulent *Bact. aertrycke* for the test inoculations. The results were in entire accord, and showed that the 13-times-vaccinated mice were much less resistant than those that received 6 or 8 injections, and certainly no more resistant than those inoculated once.

Columns 6 and 9 of Table I show a waxing and waning of resistance with repeated inoculation that can hardly be due to chance.

Since it seemed of interest to determine whether or not the waning of immunity associated with multiple injections of a bacterial vaccine was specific, or was due to some general deterioration in the resisting power of the mice, 40 13-times-vaccinated mice and 40 normal controls were injected intraperitoneally with 1000 *Pasteurella muriseptica*. The results are shown in the bottom row of Table I. The time to death of the mice that died is insignificantly

longer among the controls than among the vaccinated. The percentage of survivors is slightly higher among the controls than among the vaccinated, but here again the difference is insignificant. The proportion of completely resistant mice—those that survived for 28 days and from whose tissues *Pasteurella muriseptica* was not isolated—is insignificantly greater among the vaccinated than among the controls. There is thus no evidence that the multiple injections of *Bact. aertrycke* vaccine had lowered the general, non-specific resistance of the mice receiving them.

It seems fair to conclude that the effect of these repeated injections was to induce a specific increase in resistance to a relatively high level, followed by a specific decrease to a point at which the residual immunity was of a very low grade.

Any generalized conclusions would, of course, be quite unjustifiable. The sequence of events observed must be taken as applying to the particular conditions of this series of experiments, but not necessarily to any others. Different doses, or different intervals between successive inoculations, might have given quite different results. Nor can we answer at the moment the question that obviously presents itself—whether or not recurrent subclinical natural infections may sometimes cause a specific lowering of an acquired active immunity, instead of increasing it to a higher level.

Still less is it possible to hazard any opinion as to the probable mechanism of the decrease in immunity associated with repeated injections of a bacterial vaccine. Clearly the effect might be due to an accumulation within the tissues of the active antigenic substance on which the induction of immunity depends; or it might result from the accumulation of some antigenically related but not identical substance, such as a bacterial hapten; or it might be a secondary, but still specific, effect due to some unrelated constituent of the bacterial cell; or it might result from a specific sensitization of the tissues to one or another of these constituents.

A suspension of bacterial cells is an exceedingly crude reagent. The successful analysis of such a phenomenon as that described would seem to demand the preliminary separation of the active substance, or substances, concerned.

I should wish to express my thanks to Mrs. Joyce Wilson for her help in these experiments.

REFERENCES.

Greenwood, M., and Topley, W. W. C.—(1925) *J. Hyg.*, **24**, 45.

Greenwood, M., Newbold, E. M., Wilson, J., and Topley, W. W. C.—(1926) *Ibid.*, **25**, 336.—(1928) *Ibid.*, **28**, 127.—(1930) *Ibid.*, **30**, 240.

Greenwood, M., Topley, W. W. C., and Wilson, J.—(1931, *a*) *Ibid.*, **31**, 257.—(1931, *b*) *Ibid.*, **31**, 484.

Topley, W. W. C.—(1921) *Ibid.*, **21**, 103.

Topley, W. W. C., Ayrton, J., and Lewis, E. R.—(1924) *Ibid.*, **23**, 223.—(1925) *Ibid.*, **23**, 421.

FURTHER OBSERVATIONS ON THE ELECTRIC CHARGE OF THE ERYTHROCYTES IN CERTAIN PROTOZOAL DISEASES.

H. C. BROWN.

From the Wellcome Bureau of Scientific Research, London.

Received for publication September 29th, 1933.

In a previous communication (Brown, 1933) it has been shown that the electric charge of the erythrocytes in bird malaria bears a definite relation to the degree of infection, and that the reduction in charge of the red cells of an infected bird is apparently an important factor in the extent to which phagocytosis takes place.

Since this paper was written the electric charge of the erythrocytes of another series of 40 infected and 16 normal control birds has been studied from day to day with the same result.

For purposes of control the charge of the red cells in mice infected with *Trypanosoma equiperdum* has also been studied. It was found that whilst the infection was active, no reduction in the electric charge of the erythrocytes occurred; a distinct lowering of charge was, however, observed a few days after a curative dose of Bayer 205 had been administered; this coincides with the date of the appearance of antibodies in the blood of the mouse, as evidenced by the adhesion test (Davis and Brown, 1927).

In Fig. 1 is shown the alterations in the electric charge of the red cells of mice infected with *T. equiperdum* after they have received a curative dose of Bayer 205. The results are here expressed as the ratio of the migration times of the red cells suspended in buffered isotonic glucose solution across an arbitrary field, to the time across the same field taken by the cells of uninfected normal mice, who have also received the same dose of Bayer 205.

The mean of ten observations is taken in the case of each infected mouse, and this is compared with the mean of ten observations in two normal mice.

It will be noticed in Fig. 1 that on the fifth day after inoculation the mice were heavily infected. At this point 0·1 c.c. of a 1 per cent. solution of Bayer 205 was given subcutaneously; this dose was also given to two normal mice. Three days later there is a noticeable rise in the migration time of the red cells—that is to say, the negative charge of the cells is decreased.

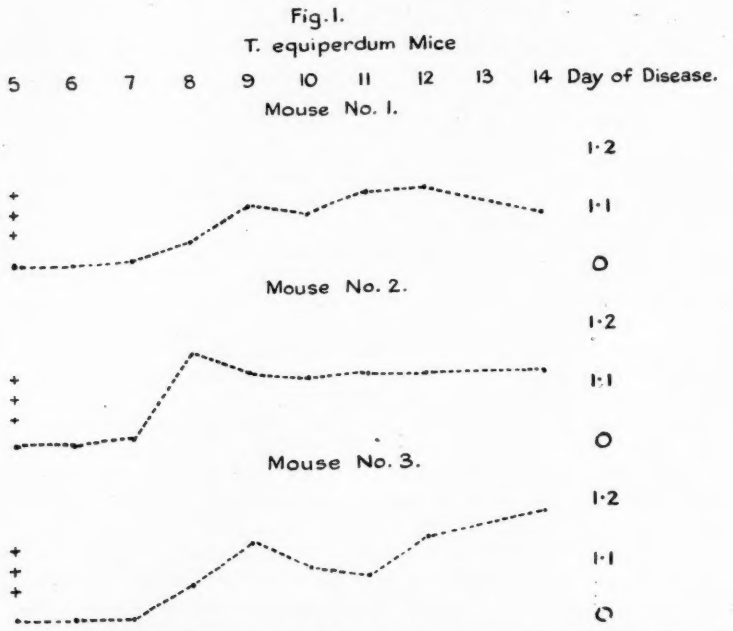
Although in the case of mice with trypanosomiasis it is unusual for the ratio of migration rate of the infected to the normal to exceed 1·15, yet in bird malaria this ratio may rise as high as 1·7.

The migration rate in trypanosome-infected mice, as in bird malaria, remains above normal for a considerable time. In certain cases of bird malaria the migration rate of the red cells was considerably above normal three months after the attack of malaria.

The way in which this change in migration rate or lowering of the electric charge of the red cells was produced in bird malaria and in trypanosome infected mice was not at first evident.

If the charge reduction could be taken to be a direct indication of the degree of immunity, then it is difficult to see how a bird which has been infected with one strain of malaria parasite, and having recovered from this has a high migration time ratio, can then be subsequently infected by a different strain of parasite.

This problem was investigated in the following manner: The red cells of a normal canary and of a canary recovering from an infection (having a



The dotted line represents the ratio of the migration times of the red cells to those of normal mice. Each mouse had a + + + infection on the 5th day after inoculation and received 0·1 c.c. of 1 per cent. solution of Bayer 205.

migration time-ratio of 1·6) were separately taken into citrated saline and subsequently washed three times in saline by means of the centrifuge. The serum of these two birds was also taken. A 20 per cent. suspension of the normal washed cells was then allowed to interact for 30 minutes at 37° C. with an equal volume of serum of the normal bird and of the immune bird respectively. A similar suspension of the washed cells of the immune bird was also allowed to react with the two above-mentioned sera.

An arbitrary volume of 30 c.mm. of the mixtures was then added to 5·c.c. of buffered 4 per cent. glucose solution, and the migration times of the red cells determined in each case.

The technique of the determination of the migration time was the same as that used in the previous communication (Brown, 1933).

The following is the result of this experiment. The figures denote in seconds the mean of ten observations of the time taken to migrate the same distance in each case. A pressure of 200 volts was applied, the current passed in each case being of the order of 740 microamperes :

Normal	cells	+	normal	serum	6·92 seconds.
,,	,,	+	immune	,,	11·99 ,,
Immune	,,	+	normal	,,	7·14 ,,
,,	,,	+	immune	,,	11·37 ,,

From this it would appear that the serum of a bird recovering from an attack of malaria is capable of increasing the migration time, *i. e.* reducing the charge of the erythrocytes of a normal bird.

If the same procedure is adopted in the case of mice which have been cured of an infection of *T. equiperdum* the same result is obtained, but in this case it is not so well marked as in the case of bird malaria, the reason being that the ratio of infected to normal migration time in the case of bird malaria was 1·6, whereas in the trypansome-infected mice it was only 1·15.

The following is the result in the case of the trypanosome-infected mice, each record being the mean of 20 observations in this experiment :

Normal	cells	+	normal	serum	6·18 seconds.
,,	,,	+	immune	,,	7·67 ,,
Immune	,,	+	normal	,,	5·87 ,,
,,	,,	+	immune	,,	7·66 ,,

Here, again, the slowing or charge-reducing action of the immune serum on the red cells is quite apparent.

So far only the charge-reducing action of the immune serum on erythrocytes of the same species has been considered ; it is now necessary to determine whether this action is in any way specific or not.

The following experiment was undertaken with this object in view.

The serum of a normal canary was allowed to interact upon the washed red corpuscles of a mouse, and similarly the serum of a canary recovered from bird malaria (having a migration time-ratio of 1·38) was also mixed with the washed red cells of the same mouse, and the migration times determined in the two cases, with the following result :

Normal canary serum	+	normal mouse cells	7·39 seconds.
Immune ,,	+	,, ,,	14·20 ,,

The effect of the same canary sera on the charge of bacteria was also investigated and the result was as follows :

Normal canary serum	+	washed bacteria	9·30 seconds.
Immune ,,	+	,, ,,	19·0 ,,

There seems to be little doubt, therefore, that the reduction of the electric charge of the erythrocytes of a bird recovering from malaria and of the cells of mice cured of an infection of *T. equiperdum* is due to some action of the serum in question; moreover, this charge-reducing action is in no way specific.

During the course of these experiments it was repeatedly observed that, when a dilute suspension of the whole blood of the animal was made in buffered glucose solution, the migration rates of the red cells of different normal animals varied. For instance, the migration times of the red cells of a mouse were consistently much smaller than when the suspension was made from human blood.

It therefore appeared of interest to see whether there was any charge-reducing factor present in normal human serum.

The result was as follows:

Normal human whole blood in buffered glucose solution .	9·16 seconds
,, mouse ,, ,, ,, .	6·44 ,,
Washed human red cells	6·11 ,,
,, mouse ,, ...	5·93 ,,
Human red cells sensitized with human serum . .	13·7 ,,
Mouse ,, ,, ,, ,, ,, . . .	10·54 ,,
Human ,, ,, ,, mouse ,, . . .	7·65 ,,
Mouse ,, ,, ,, ,, ,, . . .	6·74 ,,

The above time-records represent the mean of ten observations in each instance.

It will be seen from the above that in the first place, although a suspension of human whole blood migrates considerably slower than a similar suspension of mouse whole blood, yet human and mouse red cells, when washed, migrate at approximately the same rate.

Secondly, there is present some factor in normal human serum which is capable of reducing the charge of mouse washed cells. The migration times using whole blood do not correspond with those when sensitized cells are used, as in the latter case the electrolyte content is greater owing to the cells having been sensitized in presence of normal saline.

It appeared probable that the work of Lloyd and Paul (1929), upon the serum protein changes in malaria and typhoid fever, might throw some light on the nature of this charge-reducing factor. These observers have shown that certain definite changes take place in the amounts of the various proteins present in these diseases, the most conspicuous feature of which is the marked reduction of the serum albumen with rise of euglobulin.

Lloyd (1931) has recently extended his researches to the study of the protein graphs in syphilis, and concludes that they show a marked general resemblance to those obtained in kala-azar. He has shown that in secondary syphilis there is an enormous increase in the euglobulin—as much as tenfold in some cases—and that there is a great increase in the globulin albumen ratio.

Strumia, Mudd, Mudd, Lucké and McCutcheon (1930), when studying the mechanism of opsonin and bacteriotropin action in the case of certain acid-fast

bacteria, have shown that a definite correlation exists between the phagocytosis-promoting effect of the globulin fractions of the serum and the cataphoretic velocity of the bacteria.

In a subsequent paper Lucké, Strumia, Mudd, McCutcheon and Mudd (1933) have also correlated the degree of phagocytosis by macrophages and polymorphonuclears of sensitized sheep's erythrocytes with their cataphoretic velocity.

If these protein fraction changes in the diseases under investigation are responsible for the charge-reducing action on the erythrocytes, then in syphilis we should expect to find that the serum had a charge-reducing action upon the red cells of any animal upon which it was allowed to act. A fresh syphilitic serum giving a +++ Wassermann reaction was allowed to interact for 15 minutes at 37° C. upon an equal volume of a 5 per cent. suspension of the washed red cells of a mouse, and the migration times of these cells were compared with those of similar cells which had been sensitized with fresh normal human serum.

This experiment was repeated on three separate days, and the ratio of the migration time when syphilitic serum was used to that when normal serum was used was 1·17, 1·19 and 1·23, and on a subsequent occasion, with a different sample of syphilitic serum, the ratios were 1·25 and 1·19.

It would thus appear that in bird malaria, syphilis, and in *T. equiperdum* infections in mice, there is some substance present in the serum which is capable of reducing the electric charge of the erythrocytes, not only of the animal itself, but of the cells of other animals and even bacteria.

As the fractionation of such small quantities of sera as are available from experimental mice and canaries presents certain difficulties, the charge-reducing action of the various protein fractions of normal horse-serum was next studied.

In the first place, bacteria were washed in saline and sensitized for 30 minutes with an equal volume of a 1 per cent. solution in normal saline of albumen, pseudoglobulin and euglobulin respectively. The same volume of each mixture was then suspended in normal saline, and the migration rates of the bacteria in each case were determined. The mean of ten observations was recorded in the three instances.

The results are expressed as the ratios of the migration times to the normal, *i. e.* to the bacteria suspended in normal saline, and are as follows :

$$\frac{\text{Albumen}}{\text{Control}} = 1{\cdot}3.$$

$$\frac{\text{Pseudoglobulin}}{\text{Control}} = 1{\cdot}8.$$

$$\frac{\text{Euglobulin}}{\text{Control}} = 2{\cdot}9.$$

On three other occasions when bacteria have been acted upon by a 1 per cent. solution of euglobulin in saline, the ratio of the migration times of these bacteria to others suspended in saline have been 2·7, 2·9 and 2·8.

This experiment was then repeated with bacteria which had been washed in buffered glucose solution, and the three protein fractions were also dissolved

in buffered glucose. The charge-reducing effect in this case, though in the same order as before, was less marked and was as follows :

$$\frac{\text{Albumen}}{\text{Control}} = 1{\cdot}07.$$

$$\frac{\text{Pseudoglobulin}}{\text{Control}} = 1{\cdot}5.$$

$$\frac{\text{Euglobulin}}{\text{Control}} = 2{\cdot}1.$$

Mr. C. G. Pope kindly sent me the following :

(1) Antipneumococcus Felton protein ; protein content 13·62 per cent.

(2) Antipneumococcus acid precipitate at pH 5·0 ; protein content 1·96 per cent.

(3) Antipneumococcus pseudoglobulin ; protein content 10·02 per cent.

(4) Antityphoid albumen ; protein content 10·97 per cent.

(5) Antityphoid pseudoglobulin ; protein content 15·35 per cent.

Each of the above was diluted with normal saline to a 1 per cent. protein content of these dilutions ; one volume was taken and mixed with one volume of 20 per cent. mouse washed cells. These mixtures were allowed to interact for fifteen minutes at room temperature. Of this mixture 30 c.mm. was taken into 5 c.c. of isotonic glucose buffered at pH 7·4, and the migration times of the red cells determined, with the following result :

No. 1 : 15 seconds.
No. 2 : 7·98 ,,
No. 3 : 6·63 ,,
No. 4 : 6·62 ,,
No. 5 : 6·55 ,,
Saline control : 6·54 seconds.

No. 1 : In this case the negative charge of the cells is considerably lowered ; this apparently is accounted for by the fact that Felton (1925) found that this protein had an optimal precipitation point at a hydrogen ion concentration of pH 6·8, the observations on migration times in this case being carried out at a pH of 7·4.

No. 2 : The increase in migration time in this case compared with the normal is presumably due to the euglobulin.

Nos. 3, 4 and 5 do not show any appreciable charge-reducing action, which is what would be expected from the nature of the proteins concerned.

DISCUSSION.

During an attack of bird malaria very considerable changes take place in the electric charge of the red corpuscles of the bird ; both the parasitized and unparasitized red cells in an infected bird show a similar degree of charge. When the bird shows a strong immunizing response in that it is successfully

combating its infection the negative charge of the red cells is considerably lowered, but in cases where the parasites continue to increase steadily, the charge does not markedly differ from that of the red cells in a normal bird.

Bechhold (1904) was apparently the first to demonstrate that the negative charge of bacteria when they are sensitized is reduced. This has been confirmed by Shibley (1924) and others.

Northrop and Freund (1924) have found that the potential of red corpuscles sensitized with specific antibody was reduced to a degree dependent upon the valency of the electrolyte present. By means of Bedson and Bland's method (1929), it has been shown (Brown and Broom, 1929) that on sensitization the charge of the red cells is reduced in proportion to the strength of the immune serum used.

It is well known in the case of phagocytosis of bacteria that it is when the bacteria have their negative charge reduced in presence of a suitable electrolyte and immune serum that they enter the phagocyte.

Regarding the direct relationship of the reduction of charge of the ingested particle and the amount of phagocytosis, it has been shown (Broom and Brown, 1930) that if varying concentrations of a solution of a salt of a polyvalent anion, such as potassium ferrocyanide, are added to a mixture of phagocytes and bacteria, phagocytosis will be prevented in proportion to the interference with the reduction in charge of the bacteria. The condition of the electric charge of the particle, whether it is a bacterium or a red corpuscle, can therefore be taken as being an important factor in the process of phagocytosis.

This reduction in negative charge of the red cells also takes place, but to a less extent, in the blood of mice recovering from a *T. equiperdum* infection, the change occurring at the time when antibodies appear in the peripheral blood.

It has been shown to be of a non-specific nature, and due to the action of the euglobulin fraction of the serum upon the erythrocytes concerned.

In this connection the work of Coulter (1922) on hæmagglutinin is of interest. He found that the optimum pH for the agglutination of red corpuscles when suspended in sucrose solution was 4·75, but when sensitized it was 5·3, which is the iso-electric point of the euglobulin of the immune serum; in this case the antibody presumably forms a film on the surface of the cell, as Dean (1917) maintains occurs in the sensitization of antigen particles.

Ledingham (1907) showed that when immunizing horses against diphtheria and tetanus toxins, a horse which produced no antitoxin showed no globulin increase, whereas a horse which yielded a highly antitoxic serum showed progressive increase in the globulin.

Further evidence of the direct relationship of charge and degree of phagocytosis in bird malaria is the subject of a subsequent paper.

It is interesting to note that Kanai (1923) found that serum globulin increases the phagocytosis of carbon particles.

When one takes into account the protein changes in malaria, together with the charge-reducing action of euglobulin, it would seem quite conceivable that the rationale of the treatment of general paralysis by malaria might be due to phagocytosis of the spirochæte induced by an increased euglobulin content of the serum of the patient.

In support of this suggestion, Santangelo (1930), in discussing the mechanism of the action of malaria therapy in general paralysis, states that the introduction of the malaria parasite into the organism of the paralytic causes that reactivation of the organic processes which puts them into a condition to overcome and win the struggle against the parasites lodged in the cerebral cortex.

Further, Caldwell (1930), in discussing this subject, says that the improvement which takes place is primarily due to the destruction of the *Treponema pallidum*, and has shown that not only the sera, but also the cerebro-spinal fluids of paralytic patients who have been treated with malaria, show a higher agglutinin content to the spirochæte than in the case of untreated patients.

If, then, the agglutination of the spirochæte occurs during malaria therapy, it follows that the charge of the organism is lowered, and conditions are therefore more favourable for phagocytosis to take place.

Although a considerable amount of work has been done on the sedimentation rate of the red blood-corpuscles in various morbid conditions, no very definite explanation appears to have been given to account for the mechanism of the variations that occur.

Beaumont and Dodds (1929) state : " In diseases such as cancer, an actively-growing and fungating tumour gives a greater sedimentation rate than does a slowly-growing scirrhous carcinoma. This is probably dependent upon the amount of tissue destruction and subsequent increase in fibrinogen and globulin in the blood."

Jones (1929) concluded that either an increase of fibrinogen or a decrease in the ratio of albumen to globulin was responsible for an increased sedimentation rate.

Zárday and Farkas (1931) also were of opinion that the sedimentation rate was proportional to the amount of fibrinogen or globulin in the plasma.

It therefore only seems natural that Benvenuti (1930) should find that the acceleration of the rate of sedmentation which is constantly found in general paralysis should, during the period of malaria treatment, become even more noticeable, reaching a very high sedimentation value.

Burton (1906), when studying the cataphoretic velocity of colloidal gold particles in a gold sol to which different amounts of an aluminium salt were added, found that the instability of the colloidal solution increases as the charge of the particles approaches zero ; or, in other words, that the sedimentation rate is directly related to the charge of the particles.

If we can assume that these conditions are also applicable to the laws governing the sedimentation of red corpuscles, then it would appear that in diseases such as kala-azar, malaria, syphilis and trypanosomiasis the electric charge of the red cell and consequently the sedimentation rate is dependent upon the proportion of the various proteins present in the plasma, and that such changes in the protein composition of the cerebro-spinal fluid are responsible for the lowering of the charge and consequent phagocytosis of the treponema in cases of general paralysis undergoing malaria therapy.

The work of Northrop and Freund (1924) is in support of this ; these observers found that the addition of even a low concentration of trisodium citrate to a suspension of erythrocytes confers a negative charge on the cells

and renders the suspension stable, and that with the exception of the salts of calcium and magnesium, the concentration required to agglutinate the cells is less the higher the valency of the oppositely-charged ion.

SUMMARY.

1. It has been shown that the cause of the reduction of the electric charge of the erythrocytes in bird malaria is due to the action of the serum on these cells.

2. The action of the serum on the red cells is in no way specific.

3. That the charge-rducing action of the serum is due to an increase in the euglobulin content of the serum.

4. A theory to account for the mechanism of malaria therapy of general paralysis has been advanced.

I wish to thank Dr. R. A. O'Brien and Mr. C. G. Pope for supplying me with the various proteins, and Dr. Hamilton Fairley for the syphilitic sera used in these experiments. Also Miss I. M. Bellis, Librarian to the Wellcome Bureau of Scientific Research, for translating much of the literature.

REFERENCES.

Beaumont, G. E., and Dodds, E. C.—(1929) ' Recent Advances in Medicine,' London (Churchill), p. 365.
Bechhold, H.—(1904) *Z. phys. Chem.*, **48**, 385.
Bedson, S. P., and Bland, J. O. W.—(1929) *Brit. J. Exp. Path.*, **10**, 67.
Benvenuti, M.—(1930) *Riv. sper. Fren.*, **54**, 512.
Broom, J. C., and Brown, H. C.—(1930) *Brit. J. Exp. Path.*, **11**, 305.
Brown, H. C.—(1933) *Trans. Roy. Soc. Trop. Med. Hyg.*, **26**, 515.
Idem and Broom, J. C.—(1929) *Brit. J. Exp. Path.*, **10**, 387.
Burton, E. F.—(1906) *Phil. Mag.*, **12**, 472.
Caldwell, W. A.—(1930) *Brit. J. Exp. Path.*, **11**, 1.
Coulter, C. B.—(1922) *J. Gen. Physiol.*, **4**, 403.
Davis, L. J., and Brown, H. C.—(1927) *Trans. Roy. Soc. Trop. Med. Hyg.*, **21**, 113.
Dean, H. R.—(1917) *Lancet*, **1**, 45.
Felton, L. D.—(1925) *J. Infect. Dis.*, **37**, 304.
Jones, L. R.—(1929) *J. Lab. Clin. Med.*, **15**, 209.
Kanai, T.—(1923) *Arch. ges. Physiol.*, **198**, 401.
Ledingham, J. C. G.—(1907) *J. Hyg.*, **7**, 65.
Lloyd, R. B.—(1932) *Ind. J. Med. Res.*, **19**, 1055.
Idem and Paul, S. N.—(1929) *Ibid.*, **17**, 583.
Lucké, B., Strumia, M., Mudd, S., McCutcheon, M., and Mudd, E. B. H.—(1933) *J. Immunol.*, **24**, 455.
Mudd, S., Lucké, B., McCutcheon, M., and Strumia, M.—(1930) *J. Exp. Med.*, **52**, 313.
Northrop, J. H., and Freund, J.—(1924) *J. Gen. Physiol.*, **6**, 603.
Santangelo, G.—(1930) *Il Policlinico*, Sez. Prat., **37**, 16.
Shibley, G. S.—(1924) *J. Exp. Med.*, **40**, 453.
Zardáy, I., and Farkas, G.—(1931) *Z. exp. Med.*, **78**, 367.

ON THE NATURE OF THE TUMOURS INDUCED IN FOWLS BY INJECTIONS OF TAR.

JAMES McINTOSH.

From the Bland-Sutton Institute of Pathology, Middlesex Hospital, London.

Received for publication November 17th, 1933.

THE discovery of tar and its derivatives as carcinogenic agents has proved one of the most important discoveries of recent years in cancer research. In the case of mammals, and particularly of rats and mice, it is generally conceded that the tar-induced tumours conform in most details with spontaneous tumours. Recently it has been shown (Murphy, 1925 ; Peacock, 1933) that tumours can be induced in the fowl by the injection of tar or its derivatives into the breast muscle, but little evidence has been produced as to the nature of these tumours. This arises from the fact that the tumour problem in the fowl is complicated by the occurrence of the spontaneous filterable sarcoma, and by the failure to transplant in series these induced tumours. Further, the exact relationship of the filterable tumour of the fowl to mammalian tumours has been a subject of discussion for many years, mainly owing to the belief that these filterable tumours might not be true tumours, but specialized tissue reactions of the fowl against a virus infection ; to put it briefly, that the tumours were of the nature of granulomata. It is possible, therefore, that these tar-induced tumours of the fowl may not only help to elucidate the relation of mammalian and fowl tumours, but will throw new light on the virus theory of cancer.

These tar-induced tumours of the fowl are difficult to propagate, and only two have been propagated long enough to test some of their characteristics (Murphy, 1925 ; Peacock, 1933). Up to the time of my first communication on this subject (1933), there was no account of a filterable tumour having been obtained by tar injections. The histological pictures of the tar-induced tumours obtained by the different workers show that they belong to the fibro-sarcomatous group of neoplasms, and therefore present a histological picture closely approximating to that of the filterable sarcoma of birds.

The experiments which are detailed here were undertaken with the idea of studying the histology and the filterability of these induced tumours of the fowl, in order to determine their exact relationship to the spontaneous filterable sarcoma (Rous) of the fowl. In this paper I propose to detail the first four successful experiments.

In the first experiment 6 normal Plymouth Rock fowls, in the second 4 Plymouth Rock fowls which had been immunized against Rous sarcoma, in the third 5 normal Plymouth Rocks and in the fourth 5 normal Plymouth Rocks were used. In the first and second experiments each bird was injected in the breast muscle with 0·1 c.c. of a solution of tar in lard on four occasions on alternate sides ; for the first two injections a 2 per cent. solution of tar was used, and for the next two a 4 per cent. solution. Four of the normal birds of

Experiment No. 1 died, or were killed, at periods of 21 days (No. 390), 93 days (No. 389), 106 days (No. 385) and 261 days (No. 388) following the first injection, while 2 have survived for well over a year. In the second experiment 4 Plymouth Rock fowls, which had been rendered immune by previous injections of a non-infective mixture of Rous virus and immune serum, were used. All the control birds which received Rous filtrate with normal serum rapidly succumbed to the infection. A period of four months was allowed to elapse between the immunization and the injection of the tar. Subsequent injections showed that birds similarly treated had been rendered immune. The immunized birds died at intervals of 43 days (No. 142), 105 days (No. 146), 118 days (No. 152) and 192 days (No. 148) after the commencement of the treatment.

In the third experiment the 5 Plymouth Rock fowls were injected into the breast muscle with 0·1 c.c. of a 5 per cent. solution of tar; in all, five weekly injections were made.

In the fourth experiment 6 Plymouth Rock birds were given two injections of 0·25 c.c. of tar at 14 days' interval. One fowl died at 21 days and 2 at 29 days, the others have survived. The early deaths in this experiment seemed to be due to the toxicity of the larger dose of tar.

The average age of the normal birds used was four months and of the immune six months.

HISTOLOGICAL CHARACTERISTICS OF THE INDUCED PRIMARY TUMOURS.

An examination of the protocols of these four experiments shows that tumours of one type or another were found in about two-thirds of the birds injected (13 out of 21). Macroscopically tumours were not found in the breast muscle in every instance, though in most cases small whitish pinhead areas were discernible in the neighbourhood of the injected tar, but, as we shall see later, there was always microscopical evidence of cellular proliferation and infiltration in the injected area. In fact proliferative changes in sites other than those injected were not an uncommon feature of the experiments. In some instances the reaction was distinctly inflammatory in type, with leucocytic accumulation and infiltration, while eosinophile cells were often present in large numbers among the tumour-cells.

Microscopically the primary tumours were fibro-sarcomatous in type, some appearing to be more of an endotheliomatous or angeio-endotheliomatous type and some being leucosarcomatous, whilst in others there was a mixture of type. A striking feature was the complexity and pleomorphism observed in the histological picture of a few of these primary tumours. Particularly noticeable was the tendency for the leucosarcomata of leukæmias to occur along with the sarcomas; but as a rule the leukæmatous elements disappeared when the tumour was successfully propagated in series. In a few birds a plain leukæmia was present. (Leukæmia is used here in a wide sense, and includes any cell derived from the hæmopoietic system, whether of erythroblastic or myeloblastic origin, as in fact both types were met with.)

The leucosarcomatous nodules (collections of leucocytic cells) were most numerous in the liver, but could be found in the muscle and in other organs as well. They consisted of closely-packed round or oval cells with a medium-sized nucleus and often with deeply-staining cell protoplasm (Pl. IV, figs. 3

and 4). In a severe leukæmia the myeloid cells were found to have infiltrated the liver sinusoids to a marked degree, producing colonies or masses of these cells.

As some of the injected animals died at a relatively early date after the commencement of the injections of tar (29 days, etc.), an opportunity was given to study the early stages of tumour formation in the muscle.

The earliest changes were seen to consist of proliferation of cells round injected tar and lard or the droplets of tar. In some this appears to be mainly fibroblastic in character, with leucocytic infiltration (Pl. I, figs. 1 and 2). In others the muscle sheaths in the neighbourhood of the tar show definite proliferative changes, as indicated by the presence of numerous round or elongated fibroblastic cells invading the muscle-fibres ; on cross section these cells appear to surround completely the muscle bundle (Pl. I, figs. 3 and 4). Their position definitely suggests that they are formed by proliferation of the sarcolemma sheath. This is confirmed by the fact that, as the muscle-fibre degenerates from pressure, the ring of cells condenses to form a multinucleated cell.

In the Rous-immune fowls no tumour of Rous No. 1 type was obtained, only the hard fibro-angeio-endothelioma described above.

HISTOLOGY OF THE TRANSPLANTED TUMOURS.

So far four tar-induced tumours have been propagated in series, giving five histological types :

Tumour No. I (Fowl No. 388/32).—This tumour, in subsequent passages,

---

EXPLANATION OF PLATES.

PLATE I.

FIG. 1.—Early reaction round a deposit of tar. (Fowl No. 187/33.) × 75.
FIG. 2.—The same showing cellular infiltration. × 540.
FIG. 3.—Early reaction in injected muscle. Note spaces formerly occupied by tar and lard injection, and proliferation of cells. (Fowl No. 390/32.) × 120.
FIG. 4.—The same showing intimate relation of cells with muscle-fibres. × 540.

PLATE II.

FIG. 1.—Section of Tumour I, in first passage, showing whorls of closely packed fibroblasts. (Fowl No. 136/32.) × 120.
FIG. 2.—The same. × 540.
FIG. 3.—Section of Tumour II, showing loose arrangement of tumour. (Fowl No. 31/33.) × 120.
FIG. 4.—High power of above, showing characteristic irregularly shaped cells of the tumour. × 540.

PLATE III.

FIG. 1.—Section of Tumour III, showing the invasion of muscle with angeiomatous elements. (Fowl No. 146/33.) × 120.
FIG. 2.—High power of above, showing angeiomatous nature of the tumour. × 540.
FIG. 3.—Spindle-celled tumour showing some attempt at vessel formation. (Fowl No. 520/32.) × 60.
FIG. 4.—The same. × 270.

PLATE IV.

FIG. 1.—Section of Tumour V, showing loose arrangement of cells. (Fowl No. 233/33.) × 120.
FIG. 2.—High power of above, showing elongated spindle-cells with processes. × 540.
FIG. 3.—Leucosarcomatous nodule in liver of Fowl No. 537/32. × 120.
FIG. 4.—The same. × 540.

PLATE I.

FIG. 1.

FIG. 2.

FIG. 3.

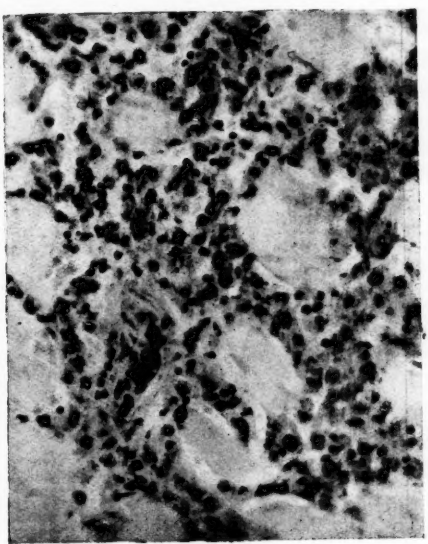


FIG. 4.

McIntosh.

PLATE II.

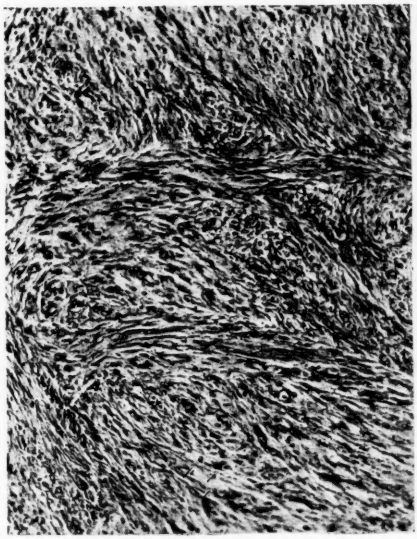


FIG. 1.

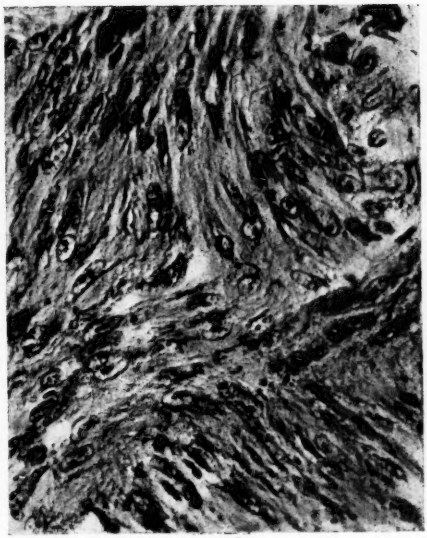


FIG. 2.

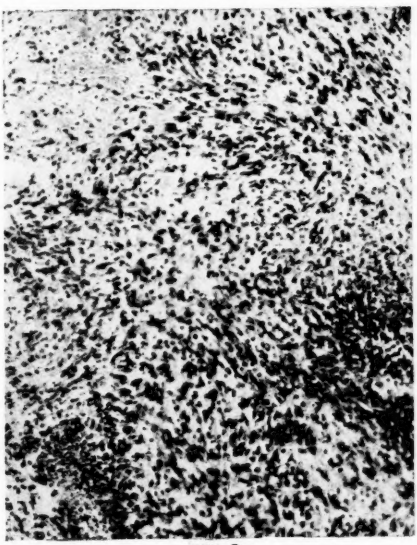


FIG. 3.

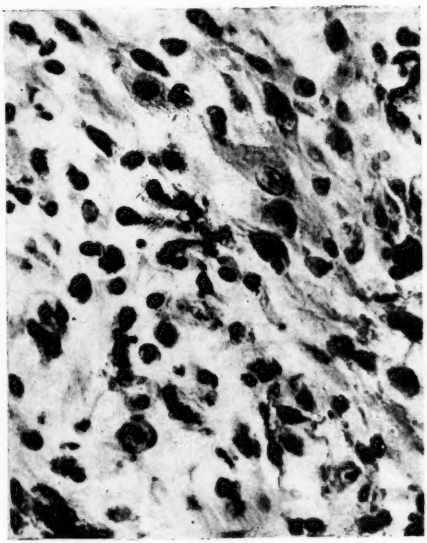


FIG. 4.

PLATE III.

FIG. 1.

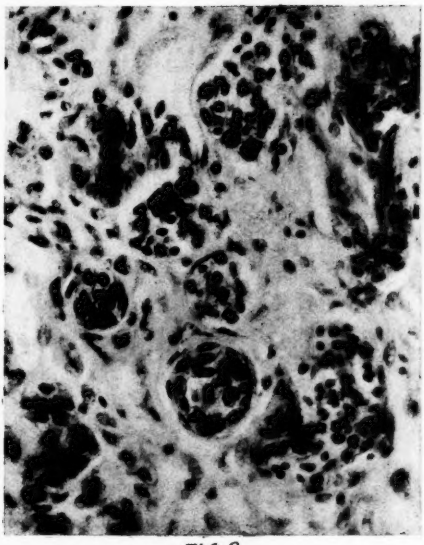


FIG. 2.

FIG. 3.

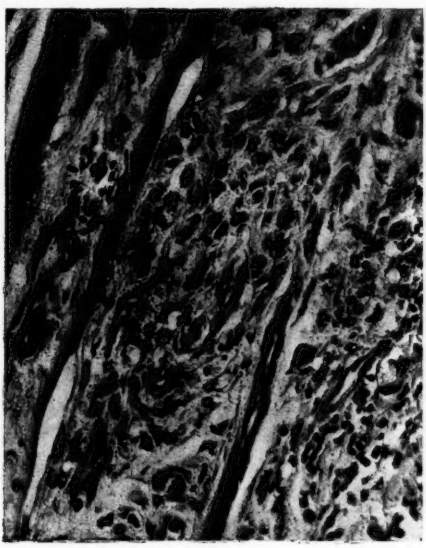


FIG. 4.

McIntosh.

PLATE IV.

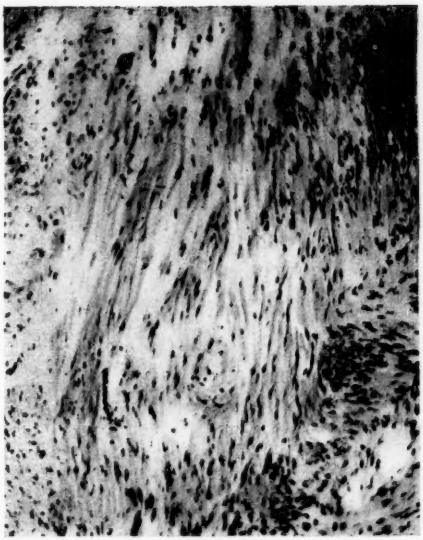


FIG. 1.

FIG. 2.

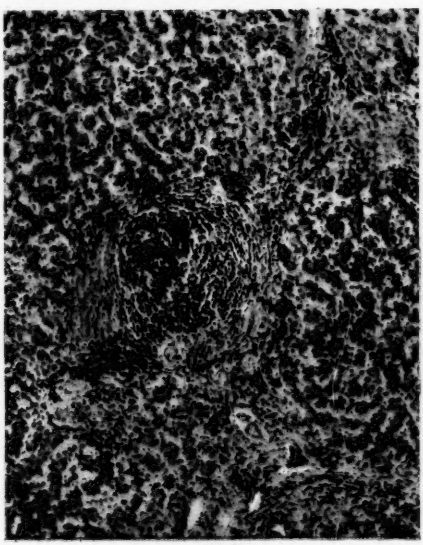


FIG. 3.

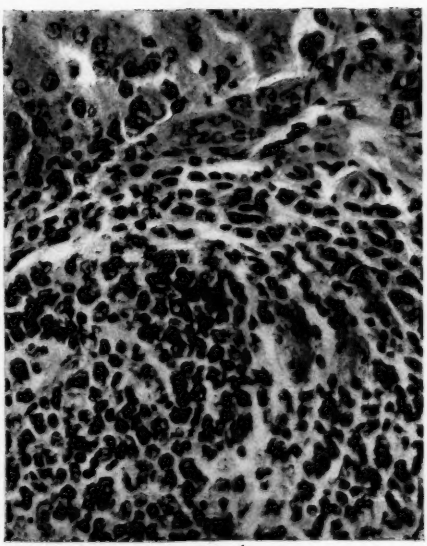


FIG. 4.

McIntosh.

is hard, slow-growing, and consists of masses of elongated cells, oval or spindle-shaped, closely packed together and with an occasional concentric arrangement of the cells. The formation of collagen fibrils is well marked. Angeiomatous or endotheliomatous elements are sparse (Pl. II, figs. 1 and 2).

Tumour No. II (Fowl No. 389/32).—This is a soft, sticky, rapidly-growing sarcoma, and, as shown in subsequent passages, composed of round, irregular shaped or spindle cells ; myxomatous changes are well marked and the formation of collagen fibrils is at a minimum (Pl. II, figs. 3 and 4). Generally this tumour differs only slightly from Rous's original sarcoma No. 1, in that the blood elements are perhaps more numerous and the myxomatous degeneration is not so extreme.

Tumour No. III (Fowl No. 146/32).—This is a very hard tumour, growing at a moderate rate. The most conspicuous feature is the presence of numerous blood-vessels, formed apparently by solid masses of endothelial cells, with a central lumen. The presence of blood in many of them indicates clearly their angeiomatous nature (Pl. III, figs. 1 and 2). There is also a good deal of fibrous and cellular intermediary tissue. This tumour shows an excessive degree of infiltration with eosinophile cells.

Tumour No. IV (Fowl No. 520/32).—This is also a hard, fibrous, slow-growing tumour, composed of elongated cells closely packed together and showing a whorled arrangement. In general the histological picture is that of Tumour No. I (Pl. III, figs. 3 and 4).

Tumour No. V.—This tumour appeared in the first passage of Tumour No. IV (see Chart No. 3), and is moderately soft. It consists of loosely-packed elongated spindles showing long fibrous processes, while the individual cells are mostly separated from one another by a clear matrix (myxomatous). In a few areas, particularly near blood-vessels, the cells are more closely packed together. Van Gieson's stain shows that there is a definite collagen matrix (Pl. IV, figs. 1 and 2).

These five tumours, therefore, seem to fall into two main groups—a sarcomatous and a fibro-angeio-endotheliomatous. The sarcomatous type has two groups—(*a*) The definite Rous type (Tumour No. II) and (*b*) the elongated spindle type (Tumour No. V). The fibro-angeio-endotheliomatous tumour seems also to be of two types : (*a*) where the blood-vessesl or angeiomatous elements are very definite (Tumour No. III), and (*b*) where the angeiomatous elements are at a minimum and the fibrous or endotheliomatous elements are well developed (Tumours Nos. I and IV).

With the exception of the tumour in Fowl No. 520/32 (Experiment 3) all the tumours seem to have bred true in subsequent passage. Charts Nos. 1, 2 and 3 show in graphic form the number of passages through which these tumours have been transmitted.

SUMMARY OF POST-MORTEM FINDINGS.

Experiment No. 1 (*Primary Tumours*).

Fowl No. 385/32.—Ill; killed 106 days after inoculation. *Macroscopical :* Breast muscle shows only a few whitish streaks in the muscle-fibre. Peritoneum full of straw-coloured fluid. Liver showed a number of hæmorrhagic

Chart No. 1.

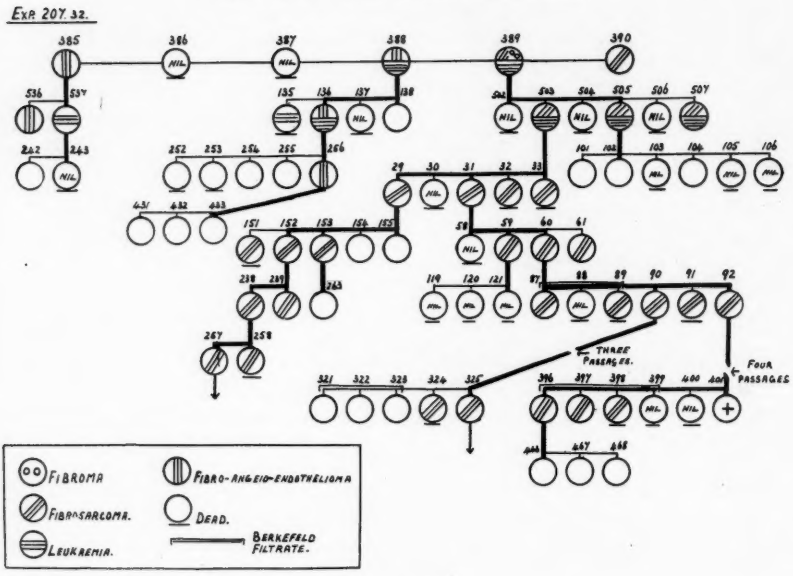


Chart No. 2.

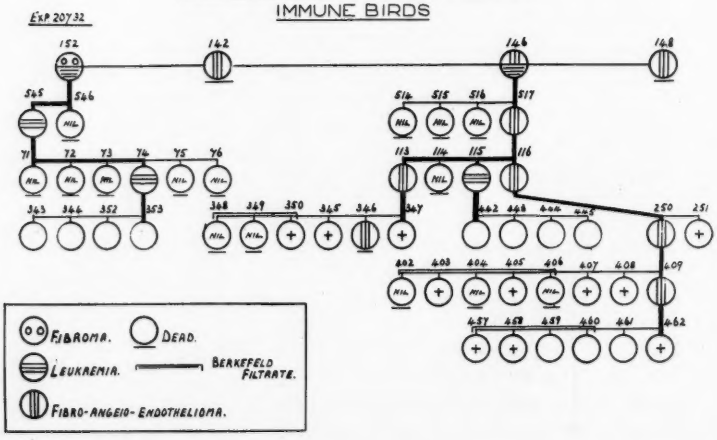
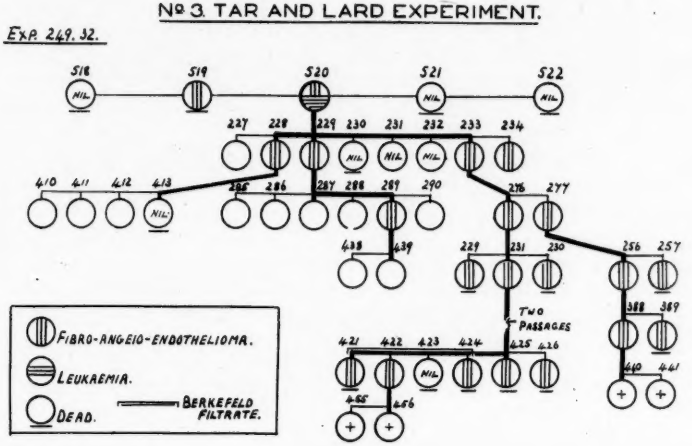


Chart No. 3.



areas. Lungs showed numerous hæmorrhagic areas. *Microscopical:* Small fibromatous areas in breast muscle, and reaction round the tar areas. Proliferative changes in lungs, with solid angio-endotheliomatous nodules with hæmorrhages. Several hæmorrhagic cystic spaces in liver; under capsule distinct endothelial proliferation with tumour-like nodules. Nothing abnormal in spleen. A good number of megaloblasts in blood.

Fowls Nos. 386/32 and *387/32.*—Killed 360 days after tar injections. No evidence of any tumour found.

Fowl No. 388/32.—Died 261 days after inoculation. *Macroscopical:* Nothing to be seen in the breast muscle. Spleen about four times the normal size. Several small whitish nodules in the heart muscle. Liver much enlarged and greyish in colour. Large white tumour nodule (1·5 cm. in diam.) in the spleen with small hæmorrhagic cyst alongside. *Microscopical:* Breast muscle not examined microscopically. Most of the liver-cells atrophied or destroyed and replaced by leukæmic cells—nothing else abnormal. Sections of the blood-vessels show a heavy leukæmia. Spleen—sinusoids distended and filled with leukæmic cells, but here and there near the trabeculæ areas of fibroblastic proliferation occur, having a tumour-like appearance, and intermingled with the leukæmic cells; numbers of eosinophile cells present. The white nodule was found to be new-growth formation consisting of definite fibro-endotheliomatous elements.

Fowl No. 389/32.—Died 97 days after inoculation. *Macroscopical:* Small black spots in breast muscle—? tar. No visible tumour. Liver somewhat enlarged, numerous small white areas, ? secondaries. *Microscopical:* "Intensive" fibroblastic reaction in the breast muscle with proliferation and cellular infiltration, including whorled areas of proliferated cells with large clear nuclei. The liver shows necrosis, with areas of cellular proliferation similar to those in breast muscle, together with leukæmic infiltration.

Fowl No. 390/32.—Died 21 days after inoculation. *Macroscopical:* Nothing abnormal seen. *Microscopical:* Section of breast muscle showed microscopic areas of infiltration and proliferation in the neighbourhood of the tar spaces with phagocytes, etc. High power: Fibroblastic changes and proliferation of muscle sheaths suggesting tumour formation.

First Passages from Experiment No. 1.

Fowl No. 592/32.—Ill; killed 147 days after inoculation. No evidence of any tumour.

Fowl No. 503/32.—Passage from Fowl No. 389/32, injected in breast muscle and peritoneum. Died 77 days later. *Macroscopical:* Nothing abnormal seen in breast muscle. Liver contained numerous small white nodules, some of which were hæmorrhagic. Kidney white in colour; appears to be a mass of growth. *Microscopical:* In the liver the small white areas are seen to consist of loosely-packed, round or elongated cells definitely of sarcomatous type—fibrosarcoma. A few areas of leucoblastic cells also present. The kidney—disorganized tubules and invaded by sarcomatous cells. Appearance somewhat suggestive of Rous cells.

Fowl No. 504/32.—Ill; killed after 77 days. No evidence of any tumour.

Fowl No. 505/32.—Died 139 days after inoculation into muscle and peritoneum from Fowl No. 389/32. *Macroscopical:* Liver greyish and mottled on surface and cut surface. Spleen and testicles normal. Greyish nodules in heart muscle. *Microscopical:* Small nodule in heart muscle composed of round or irregular-shaped sarcoma-cells. The liver shows endothelial proliferation, together with leukæmic infiltration to a marked degree. Leukæmic cells show a dark cytoplasm (? erythroleucosis).

Fowl No. 506/32.—Died some 200 days after inoculation. Nothing abnormal found.

Fowl No. 507/32.—Ill and very anæmic; killed 99 days after inoculation into breast and peritoneum. *Macroscopical:* Doubtful nodules in the spleen and liver, nothing in breast muscle. *Microscopical:* Cellular infiltration of the epicardium and heart muscle, with numerous elongated and round cells; definitely a fibro-sarcoma with leukæmic infiltration. Liver infiltrated with sarcomatous cells of Rous type.

Fowl No. 536/32.—Passage from bird No. 385/32 by grafting of liver-tissue into breast-muscle. Killed in 153 days after inoculation. *Macroscopical:* Small nodules in the spleen; nothing else seen. *Microscopical:* Section of spleen nodule shows what appears to be fibro-endotheliomatous tissue.

Fowl No. 537/32.—Sick; killed 305 days after inoculation. *Macroscopical:* Nothing abnormal seen except for some pale areas in the liver. *Microscopical:* Nothing abnormal in the breast muscle. Liver shows a number of small areas of densely-packed cells of leucosarcomatous character.

Fowl No. 135/33.—Died 80 days after inoculation. *Macroscopical:* Small whitish nodule in breast muscle. Liver greyish and mottled. Spleen normal. *Microscopical:* Leucosarcomic nodule in breast muscle, with invasion between surrounding muscle-fibres. Liver infiltrated with leukæmic cells.

Fowl No. 136/33.—Killed 62 days after inoculation. *Macroscopical:* A small tumour ($1\frac{1}{2}$ in. $\times$ $\frac{3}{4}$ in.) was present in the breast muscle with a small

cyst in the centre. Several small darkish areas in the spleen suggestive of hæmorrhages. *Microscopical:* Tumour in breast consists of a dense fibro-sarcoma with whorled endotheliomatous areas. Section of spleen showed nothing abnormal, apart from a few cyst-like hæmorrhages. Liver showed a few collections of endothelial-like cells and eosinophile cells near the blood-vessels.

Experiment No. 2 (Primary Tumours).

Fowl No. 142/32.—Macroscopical: Ill; killed 43 days after inoculation. Nothing abnormal seen in the breast muscle or internal organs. *Microscopical:* Definite areas of cellular proliferation with infiltration in the breast muscle, often directly in relation to the tar spaces. Cells chiefly elongated, with large clear nuclei. Small nodule shows angio-endotheliomatous features. Some necrosis of muscle. No evidence of leukæmia. Nothing abnormal seen in other organs.

Fowl No. 146/32.—Macroscopical: Died 105 days after inoculation. Breast muscle showed spots of tar-like substance. Large, whitish, firm nodule in the liver ($\frac{3}{4}$ in. in diameter); several smaller and less distinct ones scattered sparsely through the organ. *Microscopical:* Fibroblastic reaction in the sheath of the breast muscle with distinct tumour-like masses, mainly of fibro-angio-endotheliomatous character. There are also collections of lymphocytes apparently lying in the lymphatics, both in the sheath and in the muscle. In places the tumour-cells have spread out between the muscle-bundles. In the liver the tumour-nodules consist of leukæmic cells with fibroblastic stroma of loose elongated sarcomatous cells. There is also proliferation of an endothelial type, more or less generally throughout the liver. The spleen is distinctly leukæmic, with proliferation in the capsule.

Fowl No. 148/32.—Macroscopical: Died suddenly (192 days after inoculation). Two definite whitish areas in the breast muscle, several small hæmorrhagic nodules in the spleen. *Microscopical:* In the breast muscle small tumour, $\frac{1}{8}$ in. of closely-packed cells, in places angio-endotheliomatous in type and in others leucosarcomatous. The spleen is loosely packed with leukæmic cells. Much stroma proliferation, growth-like in appearance. The liver shows leukæmic infiltration.

Fowl No. 152/32.—Macroscopical: Died 118 days after inoculation. Slight opacity of breast muscle in two places. Liver somewhat enlarged, showing numerous whitish flakes on the surface and cut section. Spleen enlarged and very soft. Lungs œdematous. *Microscopical:* In the breast muscle small fibromatous nodules with leucocytic infiltration, but no active cellular proliferation indicative of a tumour. Large infiltration of liver with leukæmic cells. In the spleen sinusoids greatly distended and filled with leukæmic cells —some proliferation of stroma. The lungs show slight amount of collapse, no proliferation; blood-vessels contain numerous leukæmic cells.

First Passages from Experiment No. 2.

Fowl No. 514/32.—Killed 321 days after inoculation. Only some slight reaction in breast muscle. No evidence of a tumour.

Fowl No. 515/32.—Killed 140 days after inoculation. No evidence of a tumour.

Fowl No. 516/32.—Killed 321 days after inoculation. No evidence of a tumour.

Fowl No. 517/32.—Passage from experimental bird No. 146/32. Killed 140 days after inoculation with liver emulsion. *Macroscopical:* Large, hard, cartilaginous type of tumour in left breast muscle ($2\frac{1}{2}$ in $\times$ $1\frac{1}{2}$ in.). Nothing else seen. *Microscopical:* Fibrosarcoma with eosinophile infiltration, shows distinct whorl arrangement in places indicative of a fibro-endothelioma. In other places spindle-cells separated by fine collagen fibrils and clear matrix, but practically all cells are of an elongated character.

Fowl No. 545/32.—Died 103 days after passage inoculation (Berkefeld filtrate) of emulsion of spleen and liver of fowl No. 152. *Macroscopical:* Breast muscles—only one minute white spot. Liver very enlarged, greyish, with mottled appearance throughout. Spleen normal in size. Heart showed a number of white nodules on the epicardium. Testicles infiltrated with a number of nodular areas. Kidneys *nil*. *Microscopical:* Breast muscle shows some cellular accumulations—? inflammatory or leukæmic in type. Slight proliferation of muscle sheath. Heart muscle infiltrated with leukæmic cells and a number of leukæmic nodules; these cells are compressing and destroying the muscle. Other organs show infiltrations with leukæmic cells and nodular accumulations.

Fowl No. 546/32.—Killed 307 days after inoculation. *Nil* seen.

Experiment No. 3 (Primary Tumours).

Fowl No. 518/32.—Died 144 days after injection of tar. Nothing abnormal found.

Fowl No. 519/32.—Ill; killed 169 days after tar injection. *Macroscopical:* Small tumour in breast muscle ($\frac{1}{2}$ in. $\times$ $\frac{1}{4}$ in.). Also an old hæmorrhage. Other organs normal. *Microscopical:* In the breast muscle, fibrosarcoma, often with whorled endothelial areas, is invading the muscle associated with densely packed leucosarcomic nodules. The liver is mainly normal, only a few localized leukæmic collections—perhaps some proliferation of the liver stroma.

Fowl No. 520/32.—Became suddenly ill 195 days after tar injection, collapsed and died. *Macroscopical:* In the breast muscle of right side, whitish streaks suggestive of tumour. On the left side a hard fibrous tumour (2 in. $\times$ 1 in.). Lungs black with numerous hæmorrhages and growth-like nodules. Liver shows occasional raised hæmorrhagic nodules on the surface. On the under-surface two tumour nodules, the size of large peas. Spleen contained numerous small black hæmorrhagic areas. Other organs normal. *Microscopical:* Tumour in breast muscle consists mainly of an accumulation of elongated spindle-cells closely packed together; in other areas there is a whorled arrangement, indicative of a fibro-angeio-endothelioma. The liver shows moderate leukæmic infiltration. A number of fibrosarcomatous nodules seen together with a number of blood-cysts with growth in wall. Blood-vessels show a leukæmic infection. The spleen is leukæmic—numerous cystic hæmorrhagic spaces. In the lungs there is marked proliferation of endothelium,

forming in spaces distinct tumour-like masses of an angeiomatous character. Nothing abnormal found in testis.

Fowl No. 521/32.—Died 240 days after tar inoculation. No evidence of any tumour formation seen in sections.

Fowl No. 522/32.—Killed 321 days after tar inoculation. No evidence of any tumour formation.

First Passages from Experiment No. 3.

Fowl No. 227/33.—Still alive.

Fowl No. 228/33.—Killed 118 days after inoculation. *Macroscopical:* Small, hard tumour in the breast muscle ($\frac{1}{2}$ in. $\times$ $\frac{3}{8}$ in.). Whitish spots in liver. *Microscopical:* Breast muscle, tumour of fibro-endothelial type—dense cellular. In the liver areas of necrosis with endothelial proliferations.

Fowl No. 229/33.—Killed 40 days after inoculation. *Macroscopical:* Small, hard tumour extending for about 2 in. by $\frac{1}{2}$ in. along the breast muscle. Nothing seen in internal organs. *Microscopical:* Tumour consists of elongated spindle-cells closely packed together and often arranged in whorls. General appearance is that of a fibro-endothelioma.

Fowl No. 230/33.—Still alive.

Fowl No. 231/33.—Killed 118 days after inoculation. *Macroscopical:* No tumour in breast muscle. Whitish areas in liver—? necrosis. Spleen much increased in size. Small so-called " bleeding ulcer " on wing—? infection.

Fowl No. 232/33.—Died 91 days after inoculation. *Macroscopical:* Nothing in breast muscle. Whitish areas in liver. *Microscopical:* Liver shows areas of necrosis, with endothelial proliferation and leukæmic collections of cells.

Fowl No. 233/33.—Died 34 days after inoculation. *Macroscopical:* Peritoneum full of a mass of whitish gelatinous-like tumour; breast muscle—nothing seen. *Microscopical:* Tumour consisted of elongated spindle-shaped cells in a loose matrix. Areas of the tumour show numerous capillary blood-vessels; in a few places the tumour-cells appear to form the capillary spaces containing blood. May be said to be almost transitional type between Tumour No. 1 and No. 2.

Fowl No. 234/33.—Died 6 days after inoculation of spleen emulsion from bird No. 520/32. *Macroscopical:* Nothing abnormal seen. *Microscopical:* Small area of definite tumour infiltration in breast muscle of the fibrosarcomatous type. Also some small nodules of closely-packed cells, suggestive of leukæmic nodules. There are also necrotic areas of tumour with fibroblastic outgrowths, evidently part of the original inoculum.

Experiment No. 4 (Primary Tumours).

Fowl No. 178/33.—Died 29 days after inoculation. *Macroscopical:* Nothing abnormal to be seen in the breast muscle or organs. *Microscopical:* Muscles shows early tar lesions, showing tar with surrounding inflammatory fibroblastic and giant-cell reaction. In one area definite tumour formation with infiltration of muscle-fibres. Tumour fibrosarcomatous in character.

Fowl No. 179/33.—Died 21 days after inoculation. *Macroscopical:* Peritoneal cavity contained a large amount of yellow fluid. Liver small—? died

of tar toxicity. *Microscopical:* Cellular fibrosarcoma invading the breast muscle. Other organs normal.

Fowl No. 182/33.—Died 29 days after inoculation. *Macroscopical:* Nothing abnormal seen. *Microscopical:* Small nodule in heart muscle, mostly fibrous in character, but in places very cellular and growth-like in appearance, with commencing infiltration of the muscle. Consists mainly of elongated cells, but in certain areas angeio-endotheliomatous.

BIOLOGICAL CHARACTERISTICS.

Of these tar-induced tumours, four have been propagated in series to other fowls, two to the third generation and one to the eighth generation. The relative ease with which the tumours have been carried on in series is surprising in face of the difficulties reported by others. On the whole I think that the success is due to obtaining the tumour at a relatively early date, when it is actively growing, because if one waits until a large local tumour is evident, successful transplantation is less certain. Again, I have found that propagation is best carried out by tumour emulsion (prepared by grinding with sand and filtration through sand and paper pulp) rather than by grafts, and the injections made into the peritoneum as well as the breast muscle.

Tumour No. II, which has been propagated the largest number of times, is of the soft fibrosarcomatous type, with a general resemblance to the Rous sarcoma No. 1, although it is harder, and does not show the same extreme myxomatous degeneration associated with that tumour. This tumour, originally of mixed type (leukæmic elements), now seems to have completely lost any suggestion of the presence of leukæmia.

In a noticeable manner the virulence of all the tumours has increased in subsequent passages, as is shown by the increased ease of the take and the rapidity with which tumours develop. This is the case with the hard fibro-endothelioma tumour (No. III), which required, in the first passage, some 140 days for a recognizable tumour to develop; in the second passage, 77 days; in the third, 68 days; and in the fourth, 29 days.

The filterability of three different tumours (Nos. II, III and IV) has been successfully tested against Berkefeld filters (V). The viruses of tumours Nos. II and IV passed through with relative ease, but that of tumour No. III only with difficulty; in fact, success with the latter was only obtained when the tumour emulsion was treated with trypsin (Baker and McIntosh, 1927). In each filtration the filter was controlled by the addition of a broth culture of *B. prodigiosus* to the tumour emulsion previous to filtration.

DISCUSSION.

Before discussing these results, I think it is necessary to point out that the fact of obtaining tumours of one sort or another in just over two-thirds of the birds inoculated with tar at once does away with the idea that they might be spontaneous. For in the young fowls which we have used during the last seven years, we have not met with more than three or four tumours out of a

total of over 5000 fowls and about the same number of leukæmic conditions. In addition, most of the birds have been obtained from the same breeder, and have been kept in the laboratory for only a few days before being used in the actual experiment. Again, the occurrence in a few instances of tumours at sites other than those in which the injections of tar were made is of considerable interest, and may result from several causes. Thus the tar and lard injections might have been carried to new sites by the lymphatics or blood-stream, or the tumours may be a part of the diffuse tissue proliferation which, in certain fowls, is seen to follow the injection of tar, due probably to the direct absorption of the carcinogenic agent into the general circulation. Lastly, as local microscopical tumour-like proliferations were present in the breast muscle, it is possible that the macroscopic tumours were metastatic deposits.

The general histological characteristics of the tumours have already been referred to, but some remarks on the nature of their initial complexity are perhaps necessary. This complexity, most marked in the primary tumour, becomes less evident after the first passage, and by the second or third the tumour seems to have settled down into a single type. Subsequent passages have bred true, perhaps with the exception of the presence of leukæmic elements, although, as a rule, this dies out as the tumour growth becomes accelerated by passage.

This complexity of the histology of the tumour occurring in the fowl after tarring apparently runs parallel with what has been observed in the response of the fowl to the leukæmic virus. Thus, an analysis of the work of Ellermann (1921), Furth (1931, *a* and *b*) and others on leukæmias of birds shows that there is a good deal of pleomorphism in the type of disease produced by the inoculation of a leukæmic virus. Ellermann has suggested that the variant results are probably the result of different viruses, but Furth, on the other hand, is rather inclined to pleomorphism. Thus, with a single strain of leucosis which Furth propagated in a series of birds, he records that at one time or another it gave rise to a myeloid leucosis, a combination of myeloid and erythroleucosis, myeloma, sarcoma and lymphoid leucosis. More recently Oberling and Guerin (1933) found a similar pleomorphism during their propagation of a spontaneous strain of leukæmia. The virus, apprently affecting endothelial as well as fibrous structures, ultimately produced growth-like fibrous nodules, ending finally in a sarcomatous tumour. This tumour, they say, is filterable and corresponds in type with the endothelioma of Murray and Begg. The production, by tar injections, of filterable sarcomatous tumours in the fowl, which are comparable to the spontaneous tumours, may be said to bring the findings in the fowl into close agreement with those which have been obtained in mammals. In each species the tar-induced tumour closely approximates to that of spontaneous origin.

The results of these experiments are capable of interpretation in a number of ways. Of these, three only will be considered: (1) It would seem that tar injections in some manner, by stimulating cell proliferation, allow the sarcoma and leukæmic viruses to get a hold on young cells of mesoblastic origin, thereby initiating a continuous proliferation, resulting in a leukæmia or sarcoma. The early local proliferative fibroblastic reaction with tumour-like appearance

round the injected tar is well seen in the early lesions. It is, of course, necessary to presuppose that these viruses are widely distributed in the fowl world, as, for instance, is herpes febrilis in the human subject. (2) Or, it may be that the proliferative and tumour-like effects are due to one single pleomorphic virus with an affinity for any tissue of mesoblastic origin. Thus, at one time the result of its action may be a leukæmia, at another a fibrosarcoma, at another an endothelioma, and so on—the action of a virus with an affinity for any mesoblastic tissue. The work of Andrewes (1931) and others on the close affinities of the various viruses of the different fowl tumours lends additional support to this conception. (3) Lastly, the acceptance of the specific factor of Gye (1925) would suggest that this factor, derived from the tissue-cells affected by the injury, becomes linked with the common mesoblastic virus of the fowl and determines the type of tissue proliferation. It is evident from these experiments that although tar plays the chief *rôle* in the induction of these tumours, it plays no part in the maintenance of the subsequent cancer process.

CONCLUSIONS.

Tumours of sarcomatous type can readily be induced in the fowl by intramuscular injections of tar and lard.

Four tar-induced tumours have been transmitted in series in fowls by injections of tumour emulsion, and of these, three have also been transmitted by means of cell-free filtrates (Berkefeld).

Tar-induced sarcomatous tumours of the fowl conform closely in morphological and biological characteristics with the naturally occurring sarcomatous tumours of the fowl.

I wish to express my indebtedness to Mr. H. A. Barker, Senior Technical Assistant in the Department, for the great assistance he has given me in carrying out this investigation.

REFERENCES.

ANDREWES, C. H.—(1931) *J. Path. Bact.*, **34**, 91.
BAKER, S. L., AND McINTOSH, J.—(1927) *Brit. J. Exp. Path.*, **8**, 257.
ELLERMANN, V.—(1921) ' The Leucosis of Fowls,' London (Gyldendal).
FURTH, J.—(1931, *a*) *Arch. Path.*, **12**, 1 ; (1931, *b*) *J. Exp. Med.*, **53**, 243.
GYE, W. E.—(1925) *Lancet*, **2**, 109.
McINTOSH, J.—(1933) *Proc. Roy. Soc.*, B, **113**, 287.
MURPHY, J. B., AND LANDSTEINER, K.—(1925) *J. Exp. Med.*, **7**, 81.
OBERLING, C. L., AND GUERIN, M.—(1933) *Bull. Ass. franç. Étude Cancer*, **22**, 180.
PEACOCK, P. R.—(1933) *J. Path. Bact.*, **36**, 141.
STURM, E., AND MURPHY, J. B.—(1928) *J. Exp. Med.*, **47**, 493.

INDEX TO AUTHORS.

ALSTON, J. M., and LOWDON, A. S. R., studies of the skin reactions to the specific soluble substances of the pneumococcus types I and II, 1

ANDERSON, D. F., the calcium content of the cerebro-spinal fluid in normal pregnancy and in eclampsia, 155

ANDREWES, C. H., and ELFORD, W. J., observations on anti-phage sera. I: The percentage law, 367; II: Properties of incompletely neutralized phage, 376.

BEDSON, S. P., immunological studies with the virus of psittacosis, 162; observations on the developmental forms of psittacosis virus, 267

BLACK, P. T., the inactivation of insulin by normal and diabetic blood, 318

BLAKELY, A. P. L., and WILKINSON, J. F., the influence of histamine and pilocarpine on the human gastric secretion, 349

BORTHWICK, G. R., experimental observations on the toxic effects of staphylococcal filtrates introduced enterally in laboratory animals, 236

BRAIN, R. T., the relationship between the viruses of zoster and varicella as demonstrated by the complement-fixation reaction, 67

BROOM, J. C., see *Findlay, G. M.*

——— and FINDLAY, G. M., the filtration of Rift Valley fever virus through graded collodion membranes, 179

BROWN, H. C., further observations on the electric charge of the erythrocytes in certain protozoal diseases, 413

BUCKLEY, O. B., the effect of subcutaneous injections of trypsin on the blood-sugar and on insulin action, 57

——— and WILHELMJ, C. M., the effect of subcutaneous injections of trypsin on the respiratory metabolism of the dog, 63

BURNET, F. M., immunological studies with phage-coated bacteria, 93; a specific soluble substance from bacteriophages, 100; specific agglutination of bacteriophage particles, 302

DAVIES, D. R., see *Gough, J.*

DICKINSON, S., and HAVARD, R. E., the reaction of the arterial blood in cancer, 394

DUGUID, J. B., see *Gough, J.*

ELFORD, W. J., see *Andrewes, C. H.*, and *Galloway, I. A.*

——— and GALLOWAY, I. A., filtration of the virus of Borna disease through graded collodion membranes, 196

——— and TODD, C., the size of the virus of fowl plague estimated by the method of ultra-filtration analysis, 240

FILDES, P., see *Knight, B. C. J. G.*

——— GLADSTONE, G. P., and KNIGHT, B.C.J.G. the nitrogen and vitamin requirements of *B. typhosus*, 189

——— and KNIGHT, B. C. J. G., tryptophane and the growth of bacteria, 343

FINDLAY, G. M., cytological changes in the liver in Rift Valley fever, with special reference to the nuclear inclusions, 207; see *Broom, J. C.*

——— and BROOM, J. C., experiments on the filtration of yellow fever virus through "Gradocol" membranes, 391

GALLOWAY, I. A., and ELFORD, W. J., the differentiation of the virus of vesicular stomatitis from the virus of foot and mouth disease by filtration, 400

GARROD, L. P., on so-called reversed selective bacteriostasis, 182

GILROY, E., see *Mackie, T. J.*

GLADSTONE, G. P., see *Fildes, P.*

GORDON, J., and THOMPSON, F. C., the parallel action of neutral salts on the inhibition of complement and on the dispersion of gelatin, 33; the reversibility of neutral salt action on complement, 277

GOUGH, J., DUGUID, J. B., and DAVIES, D. R., the renal lesions in hypervitaminosis D: Observations on the urinary calcium and phosphorus excretion, 137

GRASSET, E., and ZOUTENDYK, A., detoxication of snake venoms, and the application of the resulting antigens to rapid methods of antivenomous vaccination and serum production, 308
GYE, W. E., and PURDEY, W. J., the infective agent in tumour filtrates: A further investigation by means of anti-sera to normal tissues, 250

HARLEY, D., hay fever. I. A study of reagin-allergen mixtures, 171
HAVARD, R. E., see *Dickinson, S.*
HEALEY, M., see *Pope, C. G.*
HENDERSON, D. W., studies on *Clostridium chauvœi*. III. Passive protection with pure "O" sera, 108
HOLMES, B. E., see *Pirie, A.*

KNIGHT, B. C. J. G., see *Fildes, P.*
——— and FILDES, P., a vitamin necessary for the growth of *B. sporogenes*: Its relation to auxin and other growth factors, 112
KREMER, M., WRIGHT, S., and SCARFF, R. W., experimental hypertension and the arterial lesions in the rabbit, 281

LOWDON, A. S. R. see *Alston, J. M.*

MCEUEN, C. S., and THOMSON, D. L., the effect of hypophysectomy on the growth of the Walker rat tumour, 384
MCINTOSH, J., on the nature of the tumour induced in fowls by injections of tar, 422
MACKIE, T. J., VAN ROOYEN, C. E., and GILROY, E., an epizootic disease occurring in a breeding stock of mice: Bacteriological and experimental observations, 132
MAEGRAITH, B. G., variation in agglutination of stock cultures of meningococci, 219; rough and smooth variants in stock cultures of meningococci, 227
MARSHALL, P. G., the gonadotropic hormones (μ-factors). V. The effect of large doses on subsequent fertility, 246
MILES, A. A., optimal proportions in agglutination: With reference to the antigenic analysis of the *Brucella* group of organisms, 43
MORGAN, W. T. J., and PETRIE, G. F., the titration of anti-pneumococcus serum (type I) in mice, 323

PETRIE, G. F., see *Morgan, W. T. J.*
PIRIE, A., and HOLMES, B. E., a study of the enzymes of the agalactia virus, 290
POLSON, C., modern tests of liver efficiency applied to experimental shale oil liver necrosis, 24; the failure of prolonged administration of iron to cause hæmochromatosis, 73; glycine tolerance in experimental shale oil liver necrosis, 125
POPE, C. G., and HEALEY, M., the influence of maltose on the growth and toxin production of *C. diphtheriæ*, 77; the surface growth and toxin production of *C. diphtheriæ*, 87
PURDEY, W. J., the heterotransfer of two filterable tumours: An investigation by means of resistance induced by embryo tissues, 9; the hetero-transfer of two filterable tumours; an investigation by means of immune sera, 260; the Fujinami myxosarcoma in ducks: Resistance to reinfection, 297; see *Gye, W. E.*
PENROSE, L. S., see *Penrose, M.*
PENROSE, M., and PENROSE, L. S., the blood-group distribution in the eastern counties of England, 160

ROSE, C. F. M., the estimation of urea by urease methods in fluoride blood, 339

SCARFF, R. W., see *Kremer, M.*
SYKES, J. F., see *Taylor, N. B.*

TAYLOR, N. B., WELD, C. B., and SYKES, J. F., observations upon the absorption of calcium in normal animals, 355

THOMPSON, F. C., see *Gordon, J.*
THOMSON, D. L., see *McEuen, C. S.*
TODD, C., see *Elford, W. J.*
TOPLEY, W. W. C., the effect of repeated injections of a bacterial vaccine, 408

VAN ROOYEN, C. E., see *Mackie, T. J.*
WELD, C. B., see *Taylor, N. B.*
WHITE, F. D., on serum bilirubin: II. The relation between the bilirubin content of serum from different types of jaundice and the icterus index of the serum after removal of proteins, 17
WHITE, P. B., the O-receptor complex of *B. proteus* X19, 145
WHITEHEAD, R., the cortical lipoid of the mouse suprarenal after unilateral suprarenalectomy, 149
WILHELMJ, C. M., see *Buckley, O. B.*
WILKINSON, J. F., see *Blakely, A. P. L.*
WRIGHT, S., see *Kremer, M.*

ZOUTENDYK, A., see *Grasset, E.*